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Culture-dependent and Culture-independent Microbial Analysis of Probiotics

Robin Temmerman

UNIVERSITEIT GENT

FACULTY OF SCIENCES

Department of Biochemistry, Physiology and Microbiology
Laboratory of Microbiology

Culture-dependent and culture-independent microbial analysis of probiotics

Robin Temmerman

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List of abbreviations

AFLP	Amplified Fragment Length Polymorphism
B.	*Bifidobacterium*
DBPC	Double-Blind Placebo-Controlled
DGGE	**Denaturing Gradient Gel Electrophoresis**
dsDNA	double-stranded DNA
E.	*Enterococcus*
EC	European Commission
EU	European Union
FAO	Food and Agriculture Organization
LAB	**Lactic Acid Bacteria**
GI-tract	**Gastro-Intestinal tract**
BCCM	Belgian Coordinated Collections of Microorganisms
CFU	**Colony Forming Units**
GRAS	Generally Regarded As Safe
L.	*Lactobacillus*
Lc.	*Lactococcus*
S.	*Streptococcus*
subsp.	subspecies
LMG	Laboratory of Microbiology
MRS	de Man, Rogosa and Sharpe
KAAAB	Kanamycin Aesculin Azide Agar Base
PCR	**Polymerase Chain Reaction**
PFGE	Pulsed Field Gel Electrophoresis
RAPD	Randomly Amplified Polymorphic DNA
rDNA	ribosomal DNA
RFLP	Restriction Fragment Length Polymorphism
SDS-PAGE	Sodium Dodecyl Sulphate - PolyAcrylamide Gel Electrophoresis
ssDNA	single-stranded DNA
WHO	World Health Organization

General Introduction

Although the exact origin of the use of certain Lactic Acid Bacteria (LAB) in food fermentations is unknown, the first recordings of possible health promoting effects related to fermented milk date from the beginning of the 20th century. Metchnikoff (1907) described the positive effect of a 'fermenting bacillus' on the microflora of the large intestine through the reduction of toxic microbial activities. Further scientific interest in such fermented milk products was slowed down by the discovery of antibiotics, although from the moment antibiotic resistance arose, the 'health-promoting' bacteria regained attention and the concept of probiotics was born (Lilley and Stillwell, 1965). At present, more and more consumers have become aware of the fact that a well-balanced and healthy nutrition contributes to a good human physical condition, which explains the strong expansion of the functional foods market (to which probiotics belong), globally representing €95 billion in the year 2000 (Weststrate *et al.,* 2002). Furthermore, the European funding for research on functional foods has evolved from €12 million in 1989 to nearly €200 million in 2002 (Lucas, 2002). The long history of safe use, commonly referred to as the GRAS (Generally Regarded As Safe) status (Holzapfel et al., 2001), combined with a variety of interesting metabolic characteristics have led to a wide range of industrial applications for numerous LAB species. In the development of human probiotics, strains belonging to the genera *Lactobacillus* and *Bifidobacterium* are amongst the most commonly used, primarily because of the perception that they are autochthonous members of the intestinal microflora. However, some of the probiotic strains currently used in dairy food industries are not of human origin and do not possess this history of safe use. Consequently, before these 'new' strains can be included into a probiotic product and become commercially available, it is recommended that profound research addressing safety and functionality of the strain in question is performed (Sanders and Huis in't Veld, 1999).

Safety of probiotic strains is of major importance and because the current state of evidence suggests that probiotic effects are strain specific, a correct identification is crucial to link a strain to a specific health effect as well as to enable accurate surveillance and epidemiological studies (Reid *et al.*, 2002). Therefore, in the past decade the scientific community has paid special attention to the correct identification of bacteria used for human consumption (Hamilton-Miller *et al.*, 1999). Due to the often indiscriminate use of antibiotics in human and veterinary medicine and in animal growth promoters, antibiotic resistance has become an increasingly common characteristic in microorganisms, and it has been suggested

that also probiotic strains should be frequently subjected to an evaluation of their antibiotic resistance profiles (Charteris *et al.*, 1998). Besides the safety aspect, several research groups have recommended that in the frame of functionality, the screening process of potential probiotic strains should involve determination of their survival capacity during Gastro-Intestinal (GI)-transit, as well as their adhesion properties to the intestinal surface, two issues that have been suggested to be an important prerequisite for probiotic action (Salminen *et al.*, 1998b).

A problem impairing all (industrial) fermentations is the contamination of the process with unknown bacteria. For instance, during the production of yoghurts or probiotic products, bacterial starter cultures are often joined by additional LAB strains to improve the organoleptic or functional properties of the end product. The slightest quantitative or qualitative shift in bacterial composition may compromise the end product quality, resulting in loss of the end-product metabolites, organoleptic properties, and functionality and/or (bacterial) composition of the product. As a result, techniques capable of monitoring the bacterial (e.g. LAB) composition of the product during (and after) the production process are currently much needed. However, this type of microbiological screening at different stages in the process line of a food product can be a very laborious task when only culture-dependent techniques are available. Clearly, on-time interventions in the production process are only possible when (complex) LAB ecosystems can be analyzed in a reliable and fast culture-independent way. The need for a profound legislation setting guidelines for quality control by manufacturers as well as by independent research groups is urgent and of immediate relevance, as indicated by the Thematic Priority ‘Food Quality and Safety’ of the European Sixth Framework Programme, having a budget of €685 million (Lucas, 2002). These guidelines should be based and checked through the use of advanced and standardized methods in order to enable comparisons of quality control on an international scale.

Objectives of this work

In order to develop a successful probiotic product with long-term marketing potential, pre-production research towards the safety and functional properties of the included probiotic strains has to be performed, as well as an efficient quality control of the product itself. **The goal of this work was to evaluate and optimise new and existing methodologies to examine the microbial aspects of probiotic product quality control**.

- A collection of commercially available European probiotic products was subjected to culture-dependent microbial analysis using whole-cell protein profiling for identification of the lactic acid bacteria (LAB) isolated on a range of culture media. In this way, a first indication was obtained about the label correctness of the products.

- A selected subset of (probiotic) isolates was screened for the presence of antibiotic resistance and for GI-transit survival capacity, in order to generate information on safety and functionality of probiotic strains.

- As an alternative for culture-dependent analysis, Denaturing Gradient Gel Electrophoresis (DGGE) was optimised for the culture-independent detection of bacteria present in probiotic products. The potential of the DGGE protocol was validated by means of a parallel culture-dependent analysis.

- As a further development, the direct identification potential of DGGE was elevated through the use of nested PCR, for its application in the taxonomic characterization of (complex) bifidobacterial communities.

- Finally, within the DGGE protocol, conventional PCR was replaced by real-time PCR as a promising quantification technique facilitating an integrated qualitative and quantitative microbial analysis of probiotic products.

Short overview of this thesis

Part 1 presents an **overview of the literature** relating to the content of this work. Firstly, an extensive discussion on the probiotic concept is given, also addressing technological, functional and safety aspects of probiotic products. The second part presents an overview of techniques used in the identification of lactic acid bacteria. Finally, the analysis of LAB ecosystems is discussed, mainly focussing on the Denaturing Gradient Gel Electrophoresis technique.

Part 2 presents the **experimental work** performed in the framework of this PhD study.

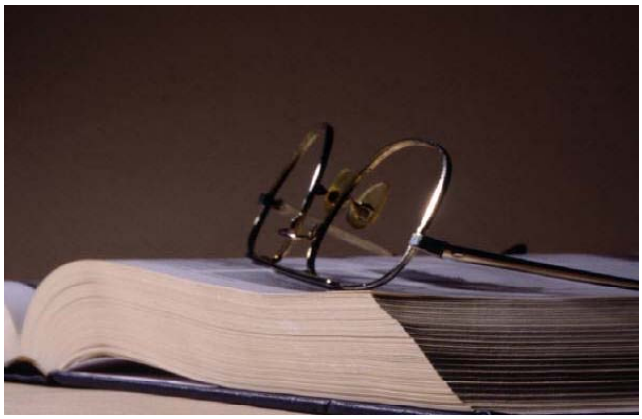
- The first chapter includes the culture-dependent microbial analysis of a range of European probiotic products. The resulting isolates were identified using SDS-PAGE separation of cellular proteins and subsequently included in trials addressing antibiotic susceptibility and some aspects of functionality (survival of GI-tract, hydrophobicity).

- The second chapter describes the optimisation of a culture-independent approach to analyse probiotic products. Denaturing Gradient Gel Electrophoresis was chosen for this purpose and a comparison of both the culture-dependent and culture-independent approaches is presented. A further optimisation of the DGGE method is also discussed, allowing the analysis of any bifidobacterial ecosystem. Finally, in order to obtain a completely culture-independent quantification of bacteria in probiotic products, the preliminary findings on the combination of real-time PCR with DGGE are presented.

Part 3 comprises the **general conclusions**, future perspectives and a summary of this work.

Part 1

Overview of the Literature



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Chapter 1
Probiotics

Before discussing the experimental work on the microbial analysis of probiotics, the first chapter provides some general information about probiotics, as well as about a number of aspects related to the production of a probiotic product, such as safety, functionality and legislation.

1.1. Definitions

At present, there are several economical, social and environmental stress factors that influence every day life of people in an increasingly negative way. In a way of counteraction, care and attention for human physical condition and health have become prominent. One of many ways to achieve such a 'good health' is through a well-balanced and healthy nutrition. In this regard, probiotic products specially designed for their health promoting properties can be part of the daily diet. Although possible health promoting properties of certain Lactic Acid Bacteria (LAB) have already been reported at the turn of the 19th century (Döderlein, 1892; Metchnikoff, 1907), it was not before the mid-1960's until the term **probiotic** was used for the first time by Lilley and Stillwell (1965) to describe substances excreted by a microorganism, which stimulate the growth of another microorganism. Over the years, numerous definitions have been proposed. Fuller (1989) described a probiotic as "*a live microbial food supplement, which beneficially affects the host by improving its intestinal microbial balance*." Further adjustments to this definition have been made in regard to discussions concerning the need for viability of the strain, the sites and modes of action, etc… This led to sometimes excessively long definitions going too much into detail. Therefore, the Food and Agriculture Organisation has recently proposed the following short definition of a probiotic: "**a live microorganism which, when administered in adequate amounts, confers a health benefit to the host**" (http://www.fao.org/es/ESN/food/foodandfood_probio_en.stm). This definition certainly does not exclude further discussion, but it clearly reflects the probiotic concept as a health-promoting microorganism.

Together with **prebiotics**, defined by Gibson and Roberfroid (1995) as "*non-digestable food ingredients, which beneficially affect the host by means of selective stimulation of growth and/or activity of one or a limited number of bacteria already present in the colon, thereby improving health of the host,*" probiotics belong to the group of functional foods. Although a clear definition of **functional food** is currently not available (Menrad *et al*., 2002), they can be described as "*modified food or food-ingredients confering a beneficial effect on health, beyond the effects of the traditional nutrients present in the food*" (ONFS Committee, 1994). Many common foods have nutritional qualities that are not recognized as recommended nutrients, but are considered to have functional benefits such as reducing risk of disease or promoting health. In this way, however, the definition of a 'functional food' is not well delineated. Because of their specific health-promoting objective, there is no doubt that probiotics are truly functional foods, partly explaining the substantial increase of the probiotic market (Stanton *et al*., 2001, Weststrate *et al*., 2002).

1.2. Lactic Acid Bacteria (LAB)

According to the definitions proposed for a probiotic, a great variety of microbial species and genera is considered to have probiotic potential (**Table 1**), most of which belong to the **Lactic Acid Bacteria**. LAB are constituted of a heterogeneous group of Gram-positive, non-sporulating, non-respiring cocci or rods, exhibiting a strictly fermentative metabolism with lactic acid as the key metabolite. The composition of this group of bacteria, originally being based on morphological and physiological descriptions, has frequently been debated due to recent phylogenetic studies. The core of LAB is composed by the genera *Lactobacillus, Leuconostoc, Pediococcus* and *Streptococcus* with taxonomical revisions and description of new genera leading to the following list of LAB genera: *Aerococcus, Alloiococcus, Carnobacterium, Dolosigranulum, Enterococcus, Globicatella, Lactobacillus, Lactococcus, Lactosphaera, Leuconostoc, Oenococcus, Pediococcus, Streptococcus, Tetragenococcus, Vagococcus* and *Weisella* (Axelsson, 1998).

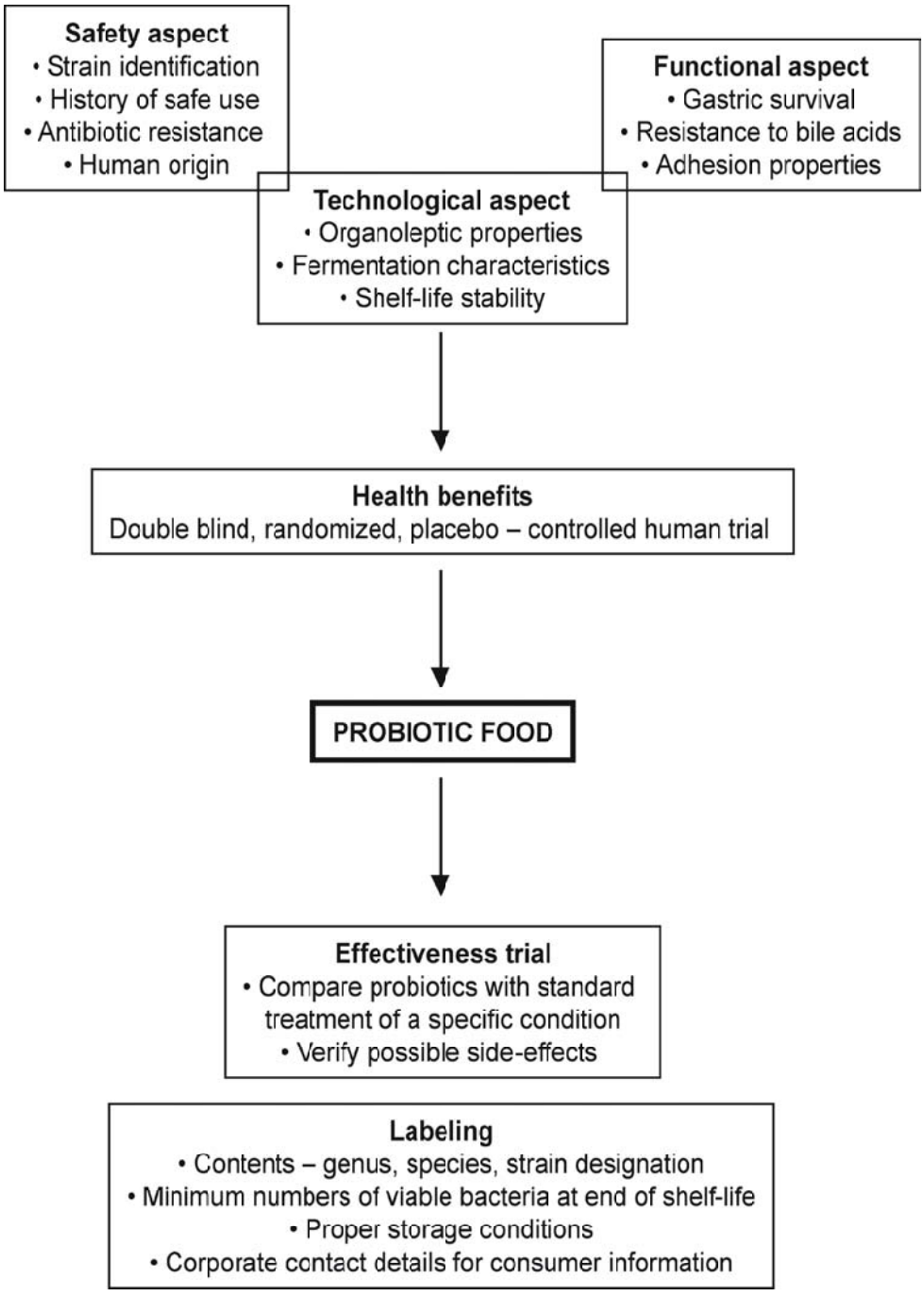
The natural habitat of these organisms includes humans, animals and plants, and their long history of safe use, commonly referred to as the GRAS (Generally Regarded As Safe) status (Holzapfel *et al.*, 2001), combined with a variety of interesting metabolic characteristics has led to a wide range of industrial applications. Flavor, texture and preservative qualities of many fermented foods such as cheese, yoghurt, sausages, sour dough breads and silage (Wood, 1998) are established through the use of species belonging to seven key LAB genera: *Lactobacillus* (milk, meat, vegetables, cereals), *Lactococcus* (milk), *Leuconostoc* (milk, vegetables), *Pediococcus* (vegetables, meat), *Oenococcus* (wine), *Enterococcus* (milk) and *Streptococcus* (milk) (Klaenhammer *et al.*, 2002). Although phylogenetically not belonging to the LAB, also the genera *Bifidobacterium, Propionibacterium* and *Brevibacterium* are used in food industries because some strains display LAB-like properties. Recent evolutions in biochemical and biotechnological techniques have also resulted in the use of LAB for the production of biopolymers and bulk enzymes, or as oral delivery vehicles (Hofvendahl & Hahn-Hagerdal, 2000; Klaenhammer *et al.,* 2002; Steidler 2002). The most important application directly targeting human health is the use of a select group of LAB species as probiotic organisms.

Table 1. Species used as probiotic (Holzapfel et al., 1998; Mercenier et al., 2002)

Lactobacillus	***Bifidobacterium***	**Other LAB**	**Non-LAB**
L. acidophilus	*B. adolescentis*	*Enterococcus faecalis*	*Bacillus cereus*
L. amylovorus	*B. animalis*	*Enterococcus faecium*	*Escherichia coli*
L. casei	*B. bifidum*	*Lactococcus lactis*	*Propionibacterium freundenreichii*
L. crispatus	*B. breve*	*Leuconostoc mesenteroides*	*(Saccharomyces cerevisiae)*
L. delbrueckii subsp. *bulgaricus*	*B. infantis*	*Pediococcus acidilactici*	*(Saccharomyces boulardii)*
L. fermentum	*B. lactis*	*Streptococcus thermophilus*	
L. helveticus	*B. longum*		
L. gallinarum			
L. gasseri			
L. johnsonii			
L. paracasei			
L. plantarum			
L. reuteri			
L. rhamnosus			
L. salivarius			

In the development of human probiotics, strains belonging to the genera *Lactobacillus, Bifidobacterium* and *Streptococcus* have been mostly used, primarily because of the perception that they are autochthonous members of the intestinal microflora (Goldin and Gorbach, 1992). In addition, these bacteria have traditionally been used in the manufacturing of fermented dairy products and have GRAS status. For instance, in a Persian version of the Old Testament (Genesis 18:8) it is stated, “Abraham owed his longevity to the consumption of sour milk,” which we now know resulted from LAB fermentation (Schrezenmeir and de Vrese, 2001). However, some of the probiotic strains currently used in dairy food industries are not of human origin and do not possess this history of safe use. Consequently, before these ‘new’ strains can be included into a probiotic product and become commercially available, profound research has to be performed addressing functionality, health benefits, safety and technological properties of the strain.

Fig 1. Guidelines for the evaluation of potential probiotic strains for food use (based on Reid *et al*., 2002).



1.3. Criteria for probiotic bacteria

While traditional starter cultures used in the dairy industry are selected for their ability to rapidly produce desirable organoleptic qualities of cultured dairy products, probiotic bacteria are selected for the potential to provide specific health or nutritional benefits following consumption. Such a selection addresses several criteria including safety, technological and functional aspects (**Fig 1**) (Saarela *et al*., 2000).

Safety aspects

The safety of probiotic strains is of major importance and guidelines for the safety assessment have been addressed in several articles (Donohue and Salminen, 1996; Salminen *et al*., 1998a; Adams, 1999). Safety aspects include the following specifications:

- The prerequisite of microbiological safety is the identification of the strain. The current state of evidence includes that probiotic effects are strain specific, meaning that a correct identification to the strain level is important to link a strain to a specific health effect as well as to enable accurate surveillance and epidemiological studies (Reid *et al*., 2002). Species discrimination of the bacteria must be established using the most current, valid methodology, preferably a combination of phenotypic and genotypic methods (**chapter 2**).

- Although recently debated, it is suggested that probiotic strains should be a normal inhabitant of the intestinal tract, taking into account the possibility that many of these bacteria exhibit host specificity (Saarela *et al*., 2000). Therefore, if a strain is to be used as a probiotic for humans, it is highly desirable that the probiotic bacterium originates from a healthy human GI- tract.

- It is advisable that bacterial species have a history of safe use in the production of fermented dairy foods, often refered to as ‘food grade’ or GRAS organisms (Holzapfel *et al*, 1998). This is important because it makes regulatory agency approval much easier. Historically, lactobacilli and bifidobacteria associated with food have been considered to be safe (Adams & Marteau, 1995). Their occurrence as normal commensals of the mammalian flora and their established safe use in a diversity of foods and supplement products worldwide support this conclusion. However, probiotics may *theoretically* be responsible for four types of side effects (Marteau, *in press*): systemic infections, deleterious metabolic activities, excessive immune

stimulation in susceptible indviduals, and (antibiotic resistance) gene transfer. In this regard, *in vitro* tests are crucial to verify several safety aspects of potential probiotic strains, for instance in order to determine the degree of enzymatic activity (bile salt deconjugation) or to test whether a strain produces a toxic compound as indicated by the EU Scientific Committee on Animal Nutrition (SCAN, 2000). SCAN guidelines mainly apply towards animal nutrition, although extrapollation to human nutrition has been made for certain aspects, mostly more rigorous.

- Due to the indiscriminate use of antibiotics in human and veterinary medicine and in animal growth promotors, antibiotic resistance has become an increasingly common characteristic in (food-borne) microorganisms (Threlfall *et al*., 2000), causing serious problems in treatment of microbial infections. Antibiotic resistance in bacteria may be intrinsic or acquired. Intrinsic resistance is a naturally occurring trait and may be considered as a species characteristic, whereas acquired resistance derives either from genetic mutations or acquisition of foreign DNA from other bacteria. Lactobacilli display a wide range of intrinsic antibiotic resistances (Danielsen and Wind, 2003), but in most cases these resistances are not of the transferable type. *Lactobacillus* strains with non-transferable antibiotic resistances do not usually constitute a safety concern. To some extent, antibiotic resistance might even be a useful property if the probiotic strain is to be used as a prophylactic agent in the treatment of antibiotic associated diarrhoea (Charteris *et al*., 1998). Although plasmid and transposon-linked antibiotic resistances are not very common among lactobacilli, they do occur (Gevers *et al*., 2003b) and their safety implications should be considered. Since transfer of antibiotic resistance genes may occur between phylogenetically distant bacteria (Courvalin, 1994), strains harboring mobile elements carrying resistance genes should be carefully assessed if they are to be used as human or animal probiotics.

- Finally, as for pharmaceutical products, human studies assessing possible side-effects should also be performed for probiotic products, as well as post-market epidemiological surveillance verifying possible adverse incidents experienced by consumers.

Technological aspects

In order to promote the consumption of probiotic products, the food industry has to respond to the demands of the consumer, meaning that all probiotic foods should be safe and functional and should possess attractive sensory properties. Before probiotic strains can be delivered to consumers, they must first be capable of being included into industrial fermentations. Subsequently, they need to survive and retain their functionality during storage as frozen or freeze dried cultures as well as in the food products into which they are commercialized. Finally, also the packaging material and storage conditions determine the quality of the probiotic product during its shelf life. Because of their long history, fermented dairy products are by far the most frequently encountered probiotic foods (Svensson, 1999). However, the use of modern technologies has also resulted in other product types such as powders, capsules and tablets, which favor oral administration in situations in which dairy based products can not be applied. Commercially available probiotic cultures may consist of a single strain or a mixture of several strains. In most cases, the probiotic properties are affected by the way in which the strain or culture has been produced (German *et al*., 1999), meaning that each strain should be characterized extensively in order to allow an effective optimization of the production process.

In case of fermented dairy products, reliable acid-forming ability is the most important characteristic in selecting starter microorganisms. When selecting probiotics, however, criteria should be related to the impact on human health and well-being. Because the environment within the GI-tract might be quite different from the environment of food, the probiotic is often not suitable as a starter organism (German *et al*., 1999). Therefore, it is quite common to use probiotic bacteria mixed together with other bacteria, e.g. *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Streptococcus thermophilus*, in order to obtain the desired flavour and texture.

Application of probiotic cultures in non-dairy products and environments represents an additional challenge in the field of viability and storage (Andersen, 1998). Since the probiotic cultures are present as additives in these kinds of products, they do not usually multiply, which sets great demands for the probiotic stability. Factors such as water activity, oxygen tension and temperature become increasingly important when dealing with these kinds of products. Storage at room temperature, which is common for several types of non-dairy probiotic products, can create an overwhelming challenge for probiotic stability. This is mostly solved by using modern encapsulation technologies, to ensure viability and stability of probiotic cultures (Myllärinen *et al*., 1998). More detailed information on different aspects of probiotic production technology can be found in the review of Saarela and co-workers (2000).

Functional aspects

Besides the technological and safety aspect, several research groups have recommended that the screening process of potential probiotics involves determination of resistance to gastric acidity and bile toxicity, adhesion to gut epithelial tissue, ability to colonize the GI-tract and ability to modulate immune responses (**Table 2**).

- Before reaching the intestinal tract, probiotic bacteria must first survive transit through the stomach, in which the secretion of gastric acid constitutes a primary defense mechanism against most ingested microorganisms. *In vitro* tests usually include the assessment of bacterial growth on MRS medium amended with hydrogen chloride to pH values between 2.0 and 3.4. However, the survival of bacterial strains in human gastric juice is a more accurate indication of the ability of strains to survive passage through the stomach, for which bifidobacteria proved less successful than lactobacilli (Dunne *et al*., 2001).

Table 2. Desired properties of a probiotic microorganism (based on Salminen *et al*., 1998a).

Desired property	Consequence
Human origin if used for human consumption	Good adaptation to human intestinal ecosystem, applicability as functional and clinical nutrition
Acid and bile resistance	Survival in the intestinal tract, preservation of adhesive and other properties
Adhesion to human intestinal cells and mucus	Immune modulation, competitive exclusion of pathogens
Production of antimicrobial substances	Pathogen inactivation and maintaining intestinal balance
Resistance to antibiotics	Combined use with antibiotics possible
Profound strain identification and characterisation	Safe use in nutritional and clinical applications
Data on minimal effective dose	Efficient clinical applications

- Besides gastric juice, probiotic strains should also possess a certain resistance against the effects of bile acids encountered in the small intestine. Bile acids are synthesized in the liver from cholesterol and are secreted from the gall bladder into the duodenum in the conjugated form (500-700 ml/day) (Hoffman *et al*., 1983). These acids then undergo extensive chemical modifications in the colon (deconjugation, dehydroxylation, dehydrogenation and deglucuronidation), almost solely as a result of microbial activity (Hylemon and Glass, 1983). Both conjugated and deconjugated bile acids exhibit antibacterial activity, however, the deconjugated forms are more inhibitory. Gram-positive bacteria are found to be more sensitive than gram-negative bacteria (Floch *et al*., 1972). Bile acid resistance, based on the relative ability to grow in the presence of bile acids, differs greatly among strains of each species of probiotic bacteria (Gilliland, 2002). Therefore, a profound selection of the most resistant strain is necessary.

- Adhesion of probiotic bacteria to the intestinal surface and the subsequent colonization of the human GI-tract have been suggested as important prerequisites for probiotic action. Adherent strains are likely to persist longer in the intestinal tract and thus have better chances to show metabolic and immunomodulatory effects than non-adhering strains (Salminen *et al*., 1996). Adhesion may also provide means of competitive exclusion of pathogenic bacteria from the intestinal epithelium. HT-29 and Caco-2 cells are human cell lines expressing morphological and physiological characteristics of normal human enterocytes that have been exploited to elucidate the mechanisms mediating enteropathogen adhesion (Cocconier *et al*., 1993). More recently, these cell lines have been used to select for and subsequently assess lactic acid bacteria on the basis of their adhesion properties (Tuomola and Salminen, 1998). **Although a lot of research effort went to probiotic adhesion studies, the role of adhesion in successful probiotic function remains speculative** (Saarela *et al*., 2000). It could also be argued that strong adhesion ability may increase the risk of infection in the host (Apostolou *et al*., 2001). Some probiotic strains are poorly adhering *in vitro* and/or *in vivo* but still show positive effects in the host. Regardless of the adhesion capacity of a probiotic strain, it is now widely accepted that administration of a daily dose of probiotics is the best way to maintain probiotic activity in the gut on a safe basis, although discussions on the quantity of this daily dose remain (Naidu *et al*., 1999).

- Besides these criteria, related to the potential of a certain strain to successfully reach the GI-tract, the main and perhaps most difficult research is determining the potential health benefits of a probiotic strain.

1.4. Health benefits and applications

After screening safety, technological properties and basic functionality of a probiotic strain, its potential health promoting properties are investigated. In general, two research tasks need to be performed in order to document probiotic activity. First and most crucial are the clinical trials analysing the beneficial effects of administering a probiotic to a group of persons. Secondly, if a certain effect has been reported, the involved microbial, biochemical and molecular mechanisms have to be unraveled. The levels of evidence for health benefits depend on the study design and the methodological quality. **The best evidence originates from randomized, double-blind, placebo-controlled (DBPC) studies monitoring a test group, which is large enough and is placed under a complete dietary supervision** (Marteau *et al*., 2002). A general recommendation for the testing of probiotic foods is that the placebo consists of the food carrier without the test probiotic. **The principle outcome of efficacy studies on probiotics should be proven benefits such as (i) statistically and biologically significant improvement in condition, symptoms, signs, well-being or quality of life; (ii) reduced risk of disease or longer period to next occurrence; (iii) faster recovery from illness. Each of these items should have a proven correlation with the probiotic tested** (Reid *et al*., 2002). Furthermore, it is also desirable that these studies are performed by independent scientific institutions and that results are published in peer-reviewed journals addressing the appropriate research fields (Ouwehand *et al*., 2002), thereby also including publications on negative results because these also contribute to the totality of the evidence supporting probiotic efficacy.

Numerous (potential) probiotic effects have been reported (Naidu *et al*., 1999; Ouwehand *et al*., 2002), some are better documented than others as a result of the complexity of the research. The strength of evidence for positive effects of probiotics in intestinal disorders appears to be most convincing in cases of antibiotic-associated diarrhoea, gastroenteritis and lactose intolerance. Evidence is increasing, although more slowly, for inflammatory bowel disease (IBD) and intestinal infections (Marteau *et al*., 2002). However, **one strain should not be expected to produce all potential benefits**. Clearly, each specific type of disorder will require a careful selection of the most suitable probiotic strain in order to achieve the optimal health benefits (Gilliland, 2002). As a result of these strain-dependent health benefits, studies are frequently performed using a mixture of multiple probiotic strains, for example in the prevention of chronic pouchitis (Gionchetti et al., 2000). A brief list of the most widely used probiotic species with their claimed probiotic action is presented in **Table 3**.

Table 3. Most widely used probiotics with their reported health benefits (based on Salminen *et al.*, 1996; Vandeplas, 1999; Ouwehand *et al.*, 2002 and Mercenier *et al.*, 2003).

Organism	Health benefit(s)
Lactobacillus acidophilus	Reduced antibiotic associated diarrhoea, immune enhancer, intestinal balance, treatment of constipation, lowering faecal mutagenity
Lactobacillus casei	Shortening of rotavirus associated diarrhoea, reduced recurrence of superficial bladder cancer, immune enhancer, intestinal balance
Lactobacillus crispatus	Immune enhancer, competitive exclusion
Lactobacillus johnsonii	Improved oral vaccination, reduced *Helicobacter pylori* infections, immune enhancer
Lactobacillus plantarum	Relief of irritable bowel syndrome, prevention of diarrhoea, reduction of LDL-cholesterol, stimulation IL-10
Lactobacillus reuteri	Shortening of rotavirus diarrhoea
Lactobacillus rhamnosus GG	Shortening of rotavirus and erythromycin-associated diarrhoea, immune enhancer, relief of inflammatory bowel disease, treatment and prevention of allergy, intestinal balance, anti-proliferative effects of cytoplasmic extracts
Bifidobacterium animalis	Immune enhancer, reduction of mucosal candidiasis
Bifidobacterium breve	Reduced symptoms of irritable bowel disease
Bifidobacterium lactis	Treatment of allergy, shortening of rotavirus diarrhoea, reduced incidence of travellers diarrhoea, improved oral vaccination, immune enhancer
Bifidobacterium bifidum	Prevention of diarrhoea, immune enhancer
Enterococcus faecium	Prevention of acute diarrhoea
Lactococcus lactis	Stimulation of interleukines
Escherichia coli	Immune enhancer
Saccharomyces boulardii	Prevention of antibiotic and *C. difficile* associated diarrhoea, prevention of traveller's diarrhoea; treatment of acute and chronic diarrhoea

- **Modulation of the intestinal microflora**. The original idea with probiotics has always been to change the composition of the normal intestinal microflora from a potentially harmful composition into a microflora that would be beneficial for the host. In general, this would mean a reduction of the number of e.g. putrefactive bacteria such as clostridia and coliforms; and an increase of lactobacilli and bifidobacteria. Due to competition for adhesion sites and nutrients, and for some strains the production of antimicrobial substances, levels of less desirable genera can be decreased. Evidence based on DBPC studies has shown that probiotics are effective against a broad range of intestinal disorders (Marteau *et al*., 2002). Although the actual mechanism is not always clear, the stabilization of the intestinal flora by probiotic strains has proven to be successful in preventing or easing the discomforts of antibiotic-associated diarrhoea, traveller's diarrhoea, gastroenteritis mainly due to rotavirus infection and intestinal infections by pathogenic bacteria (Marteau *et al*., 2001, 2002). Although the reduction of extensive growth of pathogens caused by probiotics is beneficial to the host, in some cases too much emphasis is put on this change in microflora composition without considering the actual health benefit. For some health effects it may not be necessary to obtain a measurable modification of the intestinal microflora composition (Ouwehand *et al*., 2002).

- **Immune modulation.** The intestinal mucosa provides a protective host defense against the constant presence of antigens from food and microorganisms in the gut lumen. The gut-associated lymphoid tissue (GALT) represents the largest mass of lymphoid tissue in the human body. Consequently, it constitutes an important element of the total immunologic capacity of the host. Microbial colonization begins after birth, but the development of the intestinal microflora and the gut barrier is a gradual process. The adult flora of the large intestine comprises up to 500 bacterial species (Salminen *et al*., 1998b) and it is estimated that bacteria account for 35-50% of the volume of contents in the human colon. The gut flora is an important constituent in the large intestine's defense barrier, as shown by increased antigen transport across the gut mucosa in the absence of an intestinal microflora (Isolauri *et al*., 2001). In addition to the effects of probiotics on non-immunologic gut defense (stabilization of the gut microflora), probiotic bacteria have been shown to enhance humoral immune responses and thereby promote the intestine's immunologic barrier (Isolauri *et al*., 1993). Probiotic bacteria stimulate nonspecific host resistance to microbial pathogens, and thereby aid in immune elimination (Perdigón *et al*., 1998). Probiotics have also shown to modulate the host's immune responses to potentially harmful antigens with a potential to down-regulate hypersensitivity reactions (allergy) (Sütas *et al*., 1996). The review by Isolauri and co-workers

(2001) indicates that probiotic bacteria have several immunomodulatory effects, particularly through stimulation of IgA responses, macrophage activation, cytokine production and stimulation of antigen uptake, and that these effects should be characterized during the development of clinical applications for extended target populations.

- **Lactose intolerance**. Approximately 70% of the world population suffers from a bad digestion of the disaccharide lactose. Yoghurt, other conventional starter and probiotic bacteria in fermented and unfermented milk products improve lactose digestion and eliminate symptoms of intolerance in lactose maldigesters. These beneficial effects are due to microbial β-galactosidase (lactase) in the (fermented) milk product, delayed gastrointestinal transit, positive effects on intestinal functions and colonic microflora, and reduced sensitivity to symptoms (de Vrese *et al*., 2001). Intact bacterial cell walls, which function as a mechanical protection of lactase during gastric transit, and the release of this lactase into the small intestine, are determinants of efficiency. Probiotic bacteria, mainly targeting the colon, normally promote lactose digestion in the small intestine less efficiently than yoghurt cultures do, because the better resistance of probiotics towards bile acids prevents β-galactosidase release into the small intestine. They may, however, alleviate clinical symptoms brought about by undigested lactose (de Vrese *et al*., 2001).

- Inflammatory Bowel Disease (IBD). IBD refers to disorders of unknown cause that are characterized by chronic or recurrent intestinal inflammation. Such disorders include ulcerative colitis, Crohn's disease and pouchitis (Marteau *et al*., 2001). The aetiology of the disease is currently not completely understood, but IBD may result from abnormal host responses to some members of the intestinal flora or from a defective mucosal barrier (Sartor, 1995). Several DBPC studies have recently been performed with probiotics in various IBD-related conditions, and evidence for a relevant effect is now sufficiently strong (Marteau *et al*., 2002). Administration of probiotics has demonstrated to reduce the number of relapses and prolong the period of remission (Gionchetti *et al.*, 2000; Hamilton-Miller, 2001).

- Colorectal Cancer (CRC). The role of diet in the development and occurrence of CRC is no longer under debate. Diets rich in fat and meat favour an intestinal flora mainly comprising higher numbers of putrefactive bacteria such as *Bacteroides* and *Clostridium*, and lower numbers of *Bifidobacterium* (Benno *et al*., 1991). These putrefactive bacteria enhance faecal enzyme activity (β-glucuronidase, azoreductase, urease, etc…), thereby converting pro-carcinogenic food compounds into carcinogens, which aids the development of CRC. It

should be pointed out that **there is no direct experimental evidence for cancer suppression in man as a result of consumption of probiotics.** However, there is a wealth of indirect evidence, based on laboratory results, although the precise mechanisms by which LAB may inhibit CRC are currently unknown. They might include alteration of the metabolic activities of intestinal microflora; alteration of physico-chemical conditions in the colon; binding and degrading potential carcinogens; quantitative and/or qualitative alterations in the intestinal microflora incriminated in producing putative carcinogens and promotors; production of antitumorigenic or antimutagenic compounds; enhancing the host's immune response; and effects on the physiology of the host. A review on this topic was made by Rafter (2002).

- **Miscellaneous**. Besides the main research topics on probiotic health benefits outlined above, various other applications are to be considered, such as enhancement of the host's digestion, production of vitamins, inhibition of *Helicobacter pylori* colonization, prevention of urogenital tract infections, reduction of LDL-cholesterol levels… Notwithstanding the remaining research hurdles ahead, the idea that what one ingests most likely influences the health of the gut, is conceptually appealing to many consumers. Dietary adjustments for conditions such as IBD consist largely of nutritional replenishment and correction of specific deficits with little evidence for a primary therapeutic benefit. **Therapeutic** modification of the gut flora with functional foods such as probiotics may empower patients and enable them to achieve an enchanced sense of control in the management of their illness. In this respect, functional nutrients promise to become a useful (adjunct to conventional) drug therapy (Shanahan, 2002).

Once healthful attributes of a probiotic strain have been identified, product, regulatory and labeling issues remain to be addressed prior to marketing. These issues are complicated because they differ for each country, but are likewise critical because they provide the means for communication of the product benefits for the consumer. The regulatory climate worldwide appears to be one of caution about overstating the benefits of such probiotic products, but at the same time it does not prevent corporate commitment to marketing (Sanders and Huis in't Veld, 1999).

1.5. Regulations and Quality Control

Regulatory and product labeling issues in the functional foods area (probiotics are not considered separately in this field) primarily involve two issues, safety and assuring that product labeling and promotion, while communicating healthful product effects, is not misleading. This second item involves regulation of health claims. In general, a health claim can be defined as a direct, indirect or implied claim in food labeling, advertising and promotion that consumption of a food carries a specific health benefit or avoids a specific health detriment (Sanders and Huis in't Veld, 1999). Regulations are far from unanimous worldwide and have, regarding certain aspects, evolved better for animal feed than for human nutrition (SCAN, 2000). However, recent efforts lead to the slow but certain unification of existing regulatory frameworks. A brief historic overview of regulatory and labelling issues is presented in **Table 4**.

- The concept of functional foods was first used in **Japan** in the late 1980s. Due to the fact that the market of functional foods was growing rapidly, the demand emerged to have legal support in order to control the unjustified health claims that were suggested by some products. In Japan, as in Europe today, **it was the industry that stimulated the regulator to have legal supervision of the claims**. The Japanese government, under the Nutritional Improvement Law, has provided financial grants to stimulate research on the physiological function of foods. It took the Functional Foods Discussion Group approximately four years before the Ministry of Health and Welfare issued in 1991 the 'Labeling Regulation for Foods for Specified Health Use' (FOSHU). FOSHU approval requires a collection of information, including safety reports, scientific evidence of the effects in humans, and nutritional analysis. A FOSHU product is approved by the Minister of Health and Welfare and of which has been proven to be effective in the maintenance and the improvement of health. Therefore, the approval system gives permission to make certain claims in labeling a FOSHU food and is the first regulatory system for functional foods.

Table 4. Historical overview of regulatory and labeling issues. (Deduced from Pascal, 2003; more information on http://www.functionalfoods.nu/)

1991 :

- FOSHU regulation in Japan
- 1st International Conference on nutrition and aging in Tokyo (ILSI-Japan)

1993 :

- FDA regulation on general requirements for health claims in labeling

1995 :

- 1st International Conference on East/West Perspectives on Functional Foods

1996 :

- EU concerted action : Functional Food Science in Europe (FUFOSE)

1999 :

- FUFOSE : in its final consensus document two new types of claims were proposed

2000 :

- SCAN paper : Scientific Committee on Animal Nutrition
- EU Directive 2000/13/EC on labeling
- BEUC : The European Consumer's Organisation
- EU White Paper on Food Safety (january 2000)

2001 :

- Discussion paper on Nutrition Claims and Functional Claims – SANCO/1341/2001

2002 :

- Draft Proposal for Regulation of the European Parliament and of the Council on Nutrition, Functional and Health Claims Made on Foods – Working Document (SANCO/1832/2002).
- PASSCLAIM

- In the **United States**, prior to 1990, no health statements were allowed on conventional foods, but the passage of the 1990 Nutrition Labeling and Education Act (NLEA) allowed foods to bear a health claim under the condition that the Food and Drug Administration (FDA) approved the claim. During the Joint FAO/WHO Food Standards Programme, the Codex Alimentarius Commission was created in 1963 by the FAO and World Health Organisation (WHO) to develop food standards, guidelines and related texts such as codes of practice. The main purposes of this Programme are protecting health of the consumers and ensuring fair trade practices in the food trade, and promoting coordination of all food standards work undertaken by international governmental and non-governmental organizations (http://www.codexalimentarius.net/). The FDA Modernization Act (FDAMA) of 1997 further amended the Food Drug and Cosmetic Act (FDCA) providing limited authorization of food labeling to include certain health claims without FDA pre-approval. This provision required that the claims were based on an 'authoritative statement' by a 'scientific body of the US Government with official responsibility for public health protection or research'. In addition to this class of health claims, another type of health statements is allowable on a class of food known as dietary supplements as stipulated in the Dietary Supplement Health and Education Act of 1994. This statement, termed a 'structure/function claim', relates a substance to normal, healthy functioning of the human body. Currently, probiotic lactobacilli and bifidobacteria are primarily sold in the US as dietary supplements or as components of foods, fluid milk, cottage cheese and yoghurt. As such, they belong to two separate regulatory categories: food ingredients and dietary supplements. The climate in the US has been conservative with regard to labeling of foods with health statements.

- The development of probiotics in **Europe** is a new concept for the European regulator. Past legislation did not anticipate any specific legal requirement of functional foods. Therefore, no specific category exists for functional foods or foods on the border between food and medicine. To complicate matters, interpretation is different according to member states. As the general knowledge in Europe on LAB and their role in the maintenance of health is high, industry is pushing to be able to use this knowledge in communications to consumers. Despite this, in Europe, national authorities do not always know how to deal with functional foods regarding authorization, certification or approval of labeling requirements. During the past years, however, substantial attention of the European Commission has been projected towards functional foods and health claims. In the consensus document resulting in Functional Food Science in Europe (FUFOSE) funded by the European Commission and coordinated by ILSI (International Life Science Institute) Europe, a definition of Functional Food was established.

The purpose of FUFOSE is to develop and establish a science based approach for the emerging concepts of functional food development. It states that the development of functional foods must be based on sound scientific knowledge of the target function in the body and must show that the effects are relevant to improved health or reduction of disease risk. In 1999, FUFOSE stated two new types of claims in its final consensus document, which were also included in the Codex by the FDA. In 2000, the EU Directive 2000/13/EC on labeling prohibits that the presentation and advertising of foods attributes any properties of prevention, treatment or cure of a human disease or any reference to such properties. The European Consumers Organisation (BEUC) thinks that to protect consumers from misleading information, it is necessary to establish harmonised rules for the use of claims and particularly health related claims in those countries where health claims are permitted or already common practice. In January 2000, an EU White Paper on Food Safety appeared, stating that consumers have the right to expect information on food quality and constituents that is helpful and clearly presented, so informed choices can be made. The Commission proposed consideration of whether to introduce specific provisions to govern 'nutrition claims' and 'functional claims', in order to reach the twin objective of achieving both the free movement of foodstuffs between Member States and a high level of consumer protection. Recently the PASSCLAIM project consisting of individual theme groups has been established, which has the objectives to produce a generic tool with principles for assessing the scientific support for health-related claims for foods and food components, to evaluate critically the existing schemes which assess the scientific substantiation of claims, and to select common criteria for how markers should be identified, validated and used in well-designed studies to explore the links between diet and health. It is expected that the E.U. proposal to govern "Health claims" should be adopted as soon as possible by the Member States. A special issue of the European Journal of Nutrition (2003) is intirely dedicated to health claim regulation.

Quality control

Besides legislation concerning health claims and labeling issues, effective on the pre-production phase, a suitable legislation should be established addressing quality control during and after the production process. The above section shows that regulators worldwide set priority at the health claim level, and the fact that this topic is far from established indicates that quality control legislation is still in its infancy. The acronym “GMP” (Good Manufacturing Practice) is used internationally to describe a set of principles and procedures, which, when followed by manufacturers of mainly therapeutic goods, helps to ensure that the manufactured products will have the required quality (http://www.health.gov.au/tga/docs/html/webgmp.htm). A basic tenet of GMP is that quality cannot be tested into a batch of product but must be built into each batch of product during all stages of the manufacturing process. Various Codes, Guides, Regulations relating to GMP have been published by different countries and trade blocks. For example, the European Union has published a GMP Guide for Medicinal Products. Most countries use compliance with a specified GMP requirement as the basis for licensing manufacturers of medicinal products and medical devices. This GMP is now being extrapollated towards functional food manufacturers, although no profound guidelines have been set up yet, and quality control is still completely up to the manufacturer himself.

In general, a comprehensive approach to shelf life of probiotic bacteria is tied to maintenance of efficacy. This implies knowledge of factors responsible for efficacy and how they are affected by shelf life. Assuming efficacy is tied to viability, the literature suggests that the performance of probiotic-containing products with regard to shelf life is mixed (Sanders and Huis in’t Veld, 1999). These studies provide ‘snapshot’ images of commercial products, some with little knowledge of storage history, or the analysis of a limited number of (national) products using sometimes doubtful identification methods (Reuter, 1997; Holzapfel *et al*., 1998; Hamilton-Miller, 1999 and Hoa *et al*., 2001). The need for a profound legislation setting guidelines for quality control by manufacturers as well as by independent research groups is urgent and of utmost importance as indicated by the Thematic Priority ‘Food Quality and Safety’ of the Sixth Framework Programme, having a budget of €685 million (Lucas, 2002). These guidelines should state modern standardized analysis methods in order to facilitate comparison on the international level.

Chapter 2
Identification methods for LAB

It has become clear that the food industry and gastrointestinal microbiologists require sensitive and reliable methods to identify and characterise the microbial content of foods and the host's GI-tract. Particular interest is aimed at the LAB because of: (i) the association of these organisms with health-promoting properties; (ii) their application in numerous food products as probiotics; and (iii) the requirements of legislative and industrial bodies, as well as the consumer, with respect to safety, labeling and strain integrity (McCartney, 2002). Additional areas of interest address contamination, food-borne pathogens, and any underlying microbiological basis to GI disorders, or susceptibility to such illnesses. Because of the extensive use in food fermentations and as food supplements, LAB have been thoroughly characterized because of their metabolic properties, growth performance, adaptability to industrial processes, sustainability in the end product and targeted site of action, shelf-life, etc… In addition to studies that aim to improve technological aspects, also safety and quality control are crucial and, ideally, should be performed on a frequent basis (Saarela *et al.*, 2000). In this context, reliable identification of LAB remains a point of crucial importance. Over the past decade, the scientific community has paid special attention to the correct identification of bacteria used for human consumption (Hamilton-Miller *et al.*, 1999). A problem impairing all (industrial) fermentations is the contamination of the process with unknown bacteria, resulting in loss of the end-product metabolites, organoleptic properties, functionality and/or (bacterial) composition of the product. As a result, techniques capable of monitoring the bacterial (e.g. LAB) composition of the product during and after the production process are currently much needed. At present, a broad range of identification techniques for LAB pure cultures or communities are available, all displaying differences in discriminatory power, reproducibility and workload.

2.1. Phenotypic methods

Although most industrial applications and probiotic health effects of LAB depend on the specific characteristics of a particular strain, it is not always necessary to identify bacteria as accurate as down to the strain level. An optimal balance has to be found between the desired taxonomic resolution of a certain application and the involved workload, speed and cost. Most phenotypic methods are much cheaper compared to genotypic methods; hence the popularity of commercially available miniaturized identification systems such as API or BIOLOG. Although the application of phenotypic techniques has proven to be useful for certain LAB, there is a general awareness that similar phenotypes displayed by strains do not always correspond to similar or even closely related genotypes. Consequently, there has been a shift towards genotypic characterization in order to provide more robust classification and differentiation (McCartney, 2002). Additional weaknesses of phenotypic methods include poor reproducibility, ambiguity of some techniques (largely resulting from bacterial growth), extensive logistics for large-scale investigations and poor discriminatory power. However, also genotypic characterization techniques are not without limitations and thus a polyphasic, or combined approach is prefered. **Table 5** summarizes a number of frequently applied identification techniques, discussed further on.

Table 5. Overview of techniques used for the identification of Lactic Acid Bacteria (L: Low; M: Moderate; H: High; Reprod.: Reproducibility)

Technique	Principle	Workload	Discriminatory Power	Reprod.	Reference
Phenotypic methods					
Morphological analysis	Microscopic analysis	L	Genus level or less	M	Gonzalez *et al.*, 2000
Physiological analysis	Growth characteristics, simple tests	M	Genus level of less	L	Corsetti *et al.*, 2001
Biochemical characterization	Assimilation and fermentation patterns (API, BIOLOG,…)	L	Genus or species level	M	Muyanja *et al.*, 2003
Protein profiling	Sodium Dodecyl Sulphate – PolyAcrylamide Gel Electrophoresis of cellular proteins	H	Species level	H	Leisner *et al.*, 2001
Genotypic methods					
Specific primers	PCR with group-specific primers	L	Depending on primer	H	Nomura *et al.*, 2002
Sequencing	Determination of gene sequences (16S rDNA…)	H	Genus to species level	H	Booysen *et al.*, 2002
RFLP	Restriction Enzyme Analysis (REA) of DNA or PCR amplicons	M	Species to strain level	H	Giraffa *et al.*, 2002
AFLP	Combination of REA and PCR amplification	H	Species to strain level	H	Giraffa and Neviani, 2000
RAPD-PCR	Randomly primed PCR	L	Species to strain level	L	Booysen *et al.*, 2002
Rep-PCR	PCR targeting repetitive interspersed sequences	L	Species to strain level	H	Gevers *et al.*, 2001
PFGE	REA and pulsed-field gel electrophoresis	H	Strain level	H	Ventura and Zink, 2002
Ribotyping	REA and oligonucleotide-probe detection	H	Species to strain level	H	Lyhs *et al.*, 2002
Hybridisation probes	DNA-DNA hybridisation using labeled probes	H	Genus to species level	H	Manero and Blanch, 2002
DNA-DNA	Hybridisation of DNA from two species	H	Species level	H	du Toit *et al.*, 1998

Especially in industrial or applied microbiology units, phenotypic tests are still being used on a routine basis for the identification of (food-associated) LAB. These methods include **morphological and physiological characterisation**, carbohydrate fermentation patterns and protein profiling. Gonzalez and co-workers (2000) identified 249 LAB isolates from freshwater fish and their environment using 44 morphological and physiological tests. A high percentage (90%) of the isolates could only be identified at the genus level, demonstrating the low taxonomic resolution of this labour-intensive approach. In most cases, these physiological tests are combined with the determination of **carbohydrate fermentation patterns**, using commercially available systems. Corsetti and co-workers (2001) analysed 317 presumptive LAB isolates from sourdoughs based on morphological and physiological characteristics followed by further identification using the commercial API50CHL system (BioMérieux, France). Still, only 38% of the isolates could be identified to the species level. A similar approach was used to identify 113 LAB isolates from a Ugandan traditional fermented beverage (Muyanja *et al*., 2003), which resulted in a tentative identification to the species level. Also the combined use of two carbohydrate fermentation test kits could not allocate 14 LAB isolates to known species (Wijtzes *et al*., 1997). These studies demonstrate that phenotypic methods have their limitations because of a sometimes poor reproducibility and a relatively low taxonomic resolution that often only allows differentiation on the genus level. The popularity of these methods is mainly due to the fact that no specialized equipment is required and because of the availability of an identification database, although only partly focusing on potential environmental or applied LAB.

In comparison **Sodium Dodecyl Sulphate – Polyacrylamide Gel Electrophoresis (SDS-PAGE) analysis of whole-cell proteins** has proven to be a more reliable identification method for LAB (Pot *et al*., 1994). Protein profiling has been successfully used by Leisner and co-workers (2001) to identify 64 LAB isolates from Malaysian condiment at the species level. However, the major drawback of this method is the rather high workload. In addition, SDS-PAGE of proteins lacks discriminatory power on the (sub)species level in the *Lactobacillus acidophilus* group (*L.acidophilus, L. crispatus, L. amylovorus, L. gallinarum, L. johnsonii* and *L. gasseri*) (Gancheva *et al*., 1999) and the *L. plantarum* group (*L. plantarum, L. pentosus* and *L. paraplantarum*) (Torriani *et al*, 2001). Differentiation of these organisms is performed using genomic techniques such as RAPD-PCR (Du Plessis *et al.*, 1995), as discussed in the next section.

Another phenotypic technique that has been used for identification of LAB isolates, but with limited success, is **Fatty Acid Methyl Ester (FAME) analysis** (Yeung *et al*., 2002). In order to obtain a reliable identification of LAB at the species level, multiple phenotypic techniques are often combined along the route of a polyphasic approach. In this way, strong features of one method can compensate shortcomings of another method.

2.2. Genotypic methods

The past two decades have witnessed the development of DNA-based identification and detection methods. Undoubtedly, one of the main advantages of these methods is their independence of variations in the growth conditions of the microorganisms. Genotypic techniques exhibit various levels of discriminatory power, from species level to differentiation of individual strains (typing). A group of genotypic methods is based on the principle of **Polymerase Chain Reaction (PCR)**, facilitating the selective amplification of specifically targeted DNA fragments, through the use of **oligonucleotide primers** under controlled reaction conditions. In theory, PCR primers can be designed for amplification at any taxonomic level. Kaufmann and co-workers (1997) designed primers that amplify a 16S rDNA fragment specific for bifidobacteria, which enable the genus-specific detection of bifidobacterial isolates in a food matrix. Beyond genus level, 16S rRNA based species- and group-specific primers have been designed for *Bifidobacterium adolescentis*, *B. angulatum, B. bifidum, B. breve*, the *B. catenulatum* group (*B. catenulatum* and *B. pseudocatenulatum*), and the *B. longum* group (*B. longum* and *B. infantis*), which are species commonly found in the human intestinal tract (Matsuki *et al.*, 1998). A similar approach using species-specific primers for various LAB was recently used for the identification of 543 cheese isolates (Mannu *et al*., 2002). At the intra-specific level, the PCR-based discrimination of *Lactococcus lactis* subsp. *lactis* and *Lc. lactis* subsp. *cremoris* was performed by Nomura and co-workers (2002). The highest discriminatory identification level was reached by Brandt and Alatossava (2003) for the specific detection of certain *Lactobacillus rhamnosus* strains using phage-related primers, and by Chagnaud and co-workers (2001), who designed primers directed at 6 *Lactobacillus* strains. Overall, the PCR-based detection of LAB and other bacteria mostly requires a difficult design strategy and thorough validation before a valuable set of primers is obtained. Therefore, this approach is only considered suitable if the presence or absence of well-known bacteria is to be verified.

When unknown bacterial isolates have to be identified, a powerful tool with high discriminatory power is **16S or 23S rDNA sequencing** (Vandamme *et al*., 1996). The obtained sequence is to be compared with DNA sequences stored in online databases of previously sequenced DNA, of which the most popular ones are the EMBL (http://www.ebi.ac.uk/embl/) and Genbank (http://www.ncbi.nlm.nih.gov/Genbank/) databases. Searching these databases

for corresponding sequences can be performed using a search algorithm, such as BLAST or FASTA. Booysen and co-workers (2002) sequenced the 16S rDNA for the identification of LAB isolates from a malting process at the subspecies level, whereas fecal LAB isolates exhibiting antimicrobial action against *Clostridium difficile* were identified at the species level by means of sequencing the 16S rDNA and Internal Transcribed Spacer (ITS) regions by Yoon-Jong Lee and co-workers (2003). Sequencing analysis of these two regions was also applied to identify 317 isolates from 25 wheat sourdoughs at the species level (Corsetti *et al*., 2001). Although a very powerful tool, sequencing of ribosomal genes is highly dependent on the reliability and the taxonomic coverage of the available databases. Furthermore, the degree of strain and inter-operon sequence variation may in some cases yield confusing identification results (Nubel *et al*., 1996).

Total DNA or amplicons resulting from a selective PCR reaction can also be digested by restriction enzymes, resulting in a mixture of fragments different in size. This technique is commonly referred to as **Restriction Fragment Length Polymorphism (RFLP)** analysis and is the prototype of a DNA fingerprinting method mostly used to identify isolates at the intra-specific level. Giraffa and co-workers (2002) applied RFLP analysis of protein-coding genes (β-galactosidase, lactose permease, and proline dipeptidase) for molecular typing of 35 *Lactobacillus delbrueckii* subsp. *lactis* and *L. delbrueckii* subsp. *bulgaricus* isolates used as starter cultures for dairy products. Furthermore, 46 LAB isolates from wine were analysed by RFLP to indicate the presence of *Oenococcus oeni*, a species showing no strain diversity in its RFLP patterns (Sato *et al*., 2000). The discriminatory power of these methods is very high (i.e. strain level) making them very useful for typing LAB starter cultures, of which the strain-specific properties are crucial to the production process. **Pulsed Field Gel Electrophoresis (PFGE)** employs an alternating field of electrophoresis to allow separation of the large DNA fragments obtained from restriction digests with rare-cutting enzymes. Crucial to PFGE is the extraction of intact chromosomal DNA, which may render the technique more time consuming than other fingerprinting strategies. However, since PFGE analyses large DNA fragments, representing the whole genome, this technique has superior discriminatory power, with excellent subspecies differentiation for a large number of microorganisms (McCartney, 2002). Multiple strains of the species *Lactobacillus johnsonii* were analysed with PFGE by Ventura and Zink (2002). They demonstrated the highly significant heterogeneity among all *L. johnsonii* isolates, potentially linked to their origin of isolation. A more advanced fingerprinting technique that combines PCR amplification with double restriction digest is **Amplified Fragment Length Polymorphism (AFLP)** (Janssen *et* al., 1996). Originally developed for plant systematics,

AFLP has been found to be a very useful fingerprinting technique for bacteria, allowing both species resolution and strain differentiation. AFLP has mostly been employed in epidemiological studies and in investigations aiming to distinguish virulence markers in food-borne pathogens. However, species-level discrimination has also been shown for the phylogenetically closely related species *L. pentosus, L. plantarum* and *L. pseudoplantarum* (Giraffa and Neviani, 2000).

DNA fingerprinting techniques that solely rely on PCR include **Randomly Amplified Polymorphic DNA (RAPD)** and **repetitive genomic element (rep)-PCR**. RAPD analysis utilises short arbitrary primers and low-stringency to randomly amplify DNA fragments, which are seperated to produce a fingerprint. Booysen and co-workers (2002) recently reported the use of RAPD to identify LAB isolates from a malting process. The great flexibility in primer choice offered by this method implies that it can be applied to differentiate LAB at different taxonomic levels ranging from genus to intra-specific level. However, because RAPD primers are not directed against a specific sequence, the reproducibility of the technique over a longer study period has often posed a problem (Olive and Bean, 1999). In contrast, rep-PCR specific primers amplify repetitive bacterial DNA elements such as ERIC, BOX or (GTG)5 (Versalovic *et al*., 1994). In recent studies, (GTG)5-PCR was proven to be useful for the differentiation of lactobacilli and bifidobacteria on the species, subspecies and potentially on the strain level (Gevers *et al*., 2001; Masco *et al*., 2003).

Ribotyping combines an enzymatic restriction digest with the detection of the resulting fragments by means of rDNA probes. Lyhs and colleagues (2002) applied ribotyping for the identification of 296 LAB isolates from vacuum-packed trout. To a large extent, the discriminatory power of this technique depends on the number and type of restriction enzymes and probes used. Either **fluorescent or radioactively labeled probes** can be used to hybridize with specific DNA sequences. For instance, enterococci were identified using a dot blot hybridisation method using species-specific probes (Manero and Blanch, 2002), whereas LAB isolates from wine have been identified using total genomic DNA probes (Sohier and Lonvaud-Funel, 1998). The authors also applied the same probes directly on wine samples, that were previously being fixated on a membrane, in which case the technique is referred to as **Fluorescent In Situ Hybridisation (FISH)**. A technique similar to the use of probes is the application of fluorescently labeled **monoclonal antibodies** directed against a specific species or strain, as demonstrated by Yuki and co-workers (1999) for the identification of the probiotic strain *Lactobacillus casei* Shirota. **DNA-DNA hybridization** is a method in genetics to measure

the degree of genetic similarity between DNA sequences. The technique is usually used to determine the genetic “distance” between two species. The DNA from the two species to be compared is extracted, purified and cut into short pieces. The DNA double strand is then separated by heating into two single strands, which are allowed to anneal with the DNA pieces of the other species. The more similar the DNA, the more of the pieces will anneal and form a hybrid double strand. Strands with a high degree of similarity will bind more firmly, and the energy required to separate them is determined. This method was used to select probiotic species out of 297 *Lactobacillus* isolates (du Toit *et al*., 1998).

Finally, because many LAB strains harbour plasmids varying in size and number, **plasmid profiling** is sometimes used for strain-specific identification as demonstrated for LAB isolates from Malaysian condiment (Leisner *et al*., 2001), and from fermented dry sausages (Gevers *et al*., 2003a). However, as not all LAB strains actually harbour plasmids, this method is not universally applicable. Moreover, plasmids can be acquired or lost during horizontal gene transfer events. To increase the reliability of a genotypic identification, a polyphasic combination of different techniques is sometimes desirable. Ventura and Zink (2002) characterized a collection of *Lactobacillus johnsonii* strains using a combination of multiplex PCR, rep-PCR, PFGE, AFLP and RAPD.

Chapter 3
Microbial analysis of LAB ecosystems

3.1. Introduction

The identification methods described in the previous section rely on the ability to isolate and cultivate LAB isolates from a given food or environmental sample. Because these culture-dependent approaches have shown limitations in terms of recovery rate, the set of obtained isolates may not always truly reflect the microbial composition of the sample (Ampe *et al*, 1999; Ercolini *et al*., 2001). Mainly detection of bifidobacteria impairs the reproducibility of culture-dependent techniques, because of the lack of suitable selective isolation media (Roy, 2001). As a result, culture-independent methods have been developed to circumvent the limitations of conventional cultivation for the analysis of microbial communities (Vaughan *et al*., 2002). For instance, during the production of yoghurts or probiotic products, in which bacterial starter cultures are often joined by additional LAB strains to improve the organoleptic or functional properties, the slightest quantitative or qualitative shift in bacterial composition may compromise the end product quality. The microbiological screening at different steps in the process line of a food product can, however, be a very laborious task when only culture-dependent techniques are available. Clearly, on-time interventions in the production process are only possible when complex LAB ecosystems can be analyzed in a reliable and fast culture-independent way.

The fastest culture-independent approach for the genus, species or strain specific detection of LAB in a food matrix is the use of a PCR assay applying **specific primers** on bacterial DNA extracted from the sample. However, with an increasing degree of microbial complexity of the sample, several PCR primers are needed in order to detect different LAB taxa or strains, thereby substantially increasing workload. Perhaps the main disadvantage of this approach is the fact that only ‘expected’ microorganisms will be detected, making such PCR assays of limited value in the analysis of highly complex ecosystems or samples showing a variable or unknown species composition. Tilsala-Timisjärvi and Alatossava (1997) designed

6 species-specific primerpairs targeting the 16S-23S intergenic rDNA region of the probiotic species *L. paracasei, L. rhamnosus, L. delbrueckii, L. acidophilus, L. helveticus* and *S. thermophilus*. Using a *Bifidobacterium*-specific set of PCR primers, Kaufmann and co-workers (1997) facilitated genus-specific detection of bifidobacteria in food and fecal samples.

Probing techniques are based on the hybridisation of synthetically prepared oligonucleotides to specific target sequences on bacterial DNA as it is the case for PCR primers. But instead of being intended for the amplification of DNA, they are linked to a radioactive or fluorescent label which enables the visual detection of the target after hybridisation under controlled conditions. The specificity of the probe is largely dependent on the target sequence, although the stringency of the hybridisation conditions and washing are also critical (O'Sullivan, 1999). Labeled oligonucleotide probes are employed in a number of assays including colony, dot-blot and *in situ* hybridisations (Giraffa and Neviani, 2000). The most frequently applied method using probes is **Fluorescent In Situ Hybridisation (FISH)** making use of fluorescence microscopy for the counting of fluorescently labeled bacteria**.** Using specific probes, Sohier and Lonvaud-Funel (1998) reported on a method to monitor the bacterial population in wine at different stages of vinification during storage, and to identify wine spoiling LAB. A more advanced FISH application used an array of genus-specific primers targeting *Bifidobacterium, Bacteroides, Lactobacillus/Enterococcus* and *Clostridium* to monitor the fecal flora of infants (Kirjavainen *et al*., 2001). A method that allows both quantitative and qualitative analysis of samples is **Flow Cytometric Analysis**. Bacteria in a liquid sample or suspension are fluorescently labeled using one or more specific dyes or probes after which the labeled solution is run through a flow cytometer or cell sorter, determining the identity and quantity of the bacteria (Bunthof and Abee, 2002). The main disadvantage of these probe-based methods is their high workload, preventing fast analysis. In addition, the use of primers and probes restricts the number of possible applications because of the limited number of bacteria targeted.

3.2. DGGE – method and applications

At present, Denaturing Gradient Gel Electrophoresis (DGGE) analysis is the most suitable and widely applied method to study complex bacterial communities originating from various environments (Muyzer, 1999). This PCR-based technique allows the sequence-dependent separation of a mixture of amplified DNA fragments, all identical in size, on an acrylamide gel containing a well-defined gradient of denaturing components (**Fig. 2 and 3**). In conventional agarose or acrylamide gel electrophoresis, DNA fragments are separated by size; with the electrophoretic mobility of each fragment being disproportionate with its size. In DGGE, DNA fragments of the same size are separated by their denaturing profile, i.e. how the double stranded DNA (dsDNA) becomes (partially) single stranded (ssDNA) when it is subjected to an increasingly denaturing environment. This physical denaturing of the dsDNA fragment does not proceed in a zipper-like manner, but gradually discrete portions of the fragment will denature through so-called melting domains. As a result of this ds to ss conformational change the DNA fragment's passage through the acrylamide gel, containing a gradient of increasing denaturants, is drastically slowed and eventually halted. The position in a gel where the dsDNA fragment melts and becomes ssDNA is dependent on the nucleotide sequence and %G+C content of the fragment. Different sequences will result in different origins of melting domains and as a consequence also in different positions in the

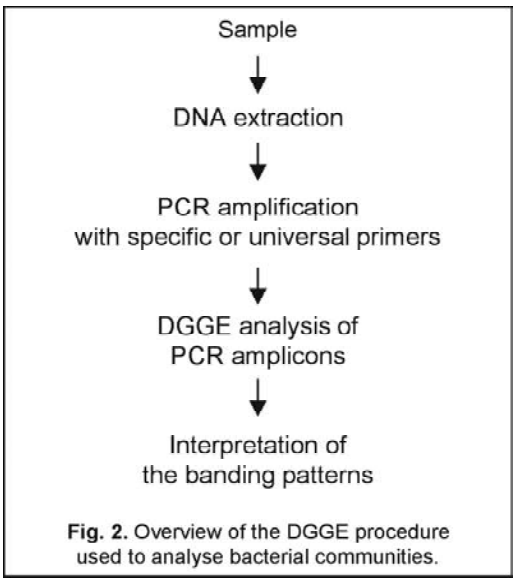


Fig. 2. Overview of the DGGE procedure used to analyse bacterial communities.

gel where the DNA fragment halts. For each kind of application the optimal denaturing gradient has to be prepared by means of mixing the desired volumes of a 100% and 0% denaturing acrylamide solution (100% denaturant is 40% formamide and 7M urea), resulting for instance in a 35-70% denaturing gel most commonly used in microbiology (Muyzer *et al.*, 1993). Originally being developed for mutation analysis, DGGE has been shown to detect differences in the denaturing behaviour of small DNA fragments (200-700bp) that differ by as little as one base pair.

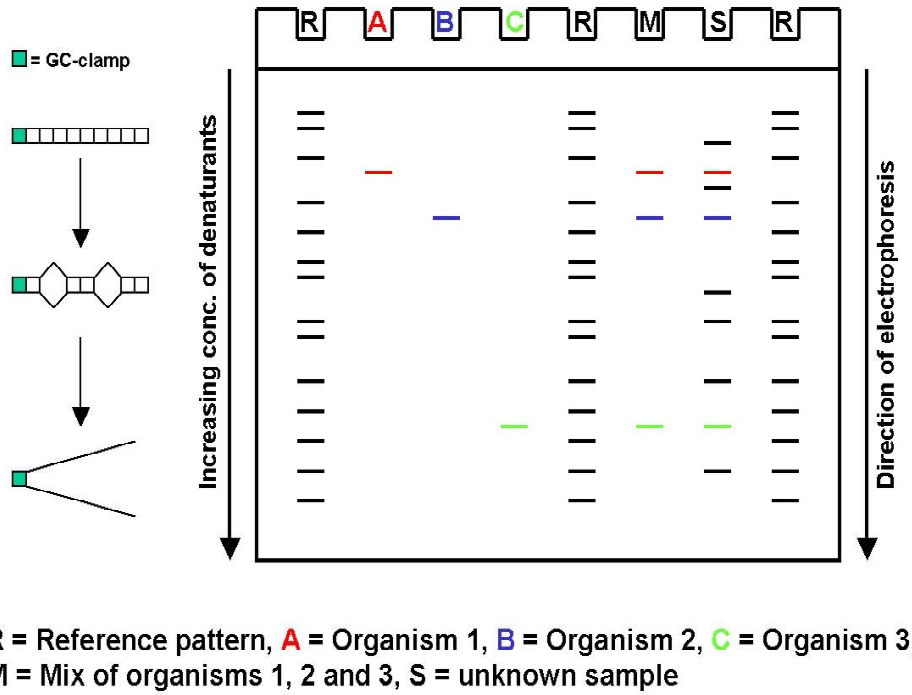


Fig. 3. Principle of DGGE. PCR amplicons of equal length are electrophoretically separated in a sequence-dependent manner. The increasing gradient of denaturing components along the gel confers the double stranded amplicons into single stranded DNA through melting domains. A GC-clamp attached to the 5' end of one of both PCR primers prevents the amplicons from completely denaturing. Different sequences will result in different origins of melting domains and as a consequence also in different positions in the gel where the DNA fragment halts.

At present, DGGE has been widely applied in various research areas including the detection of LAB in food samples and other environments. For instance, Cocolin and co-workers (2001) monitored the fermentation of Italian sausages in order to detect shifts in the involved *Micrococcaceae* population using primers targeting the V1 region of the 16S rDNA. Another study analysed cassava dough products to determine the exact composition of the microbial community (Miambi *et al*., 2002). The authors made a comparison between DGGE analysis and conventional bacterial isolation, from which they concluded that it was necessary to combine both culture-dependent and culture-independent methods to obtain a more detailed view of the microbial communities associated with indigenous cassava starch fermentations. A similar conclusion was made by Ercolini and co-workers (2001) after a comparative analysis of the bacterial composition of Mozarella cheese. The combination of DGGE analysis with Reverse Transcriptase (RT)-PCR was recently described by Randazzo and co-workers (2002) to study both the microbial composition and metabolic activity during Ragusano cheese manufacturing. Many applications of DGGE are however focussed on the study of the human or animal intestinal microflora, e.g. before, during or after the oral administration of pro- or prebiotic products (Konstantinov *et al*., 2003; Plant *et al*., 2003). General screening of the fecal flora of human neonates was performed by Favier and co-workers (2002), whereas DGGE has also been used to determine the differences in general and LAB communities between human mucosa biopsies and fecal samples (Zoetendal *et al*., 2002).

Although the above studies clearly demonstrate the broad applicability of DGGE, much of the capabilities of the technique are determined by the choice of the PCR primers. The use of universal primers (e.g. those targetting the V3 or V6-V8 region of the 16S rDNA gene) allows any bacterial community to be analysed, although in case of an ecosystem with a relatively high bacterial diversity only the dominant flora will be visualised on the DGGE gels (Zoetendal *et al*., 1998). In order to improve the detection limit for less abundant groups in the microbial ecosystem, group-specific primers need to be used. Furthermore, the use of universal primers will result in complex banding patterns with some of the bands possibly representing multiple species. Since most applications target the identification of the bands, cloning and sequencing of the extracted bands becomes necessary, which renders the DGGE method laborious and time-consuming, impairing the potential of DGGE to analyse rapidly changing and complex bacterial ecosystems. Therefore, substantial research should address the transformation of DGGE into a direct identification method, for instance towards LAB food samples.

3.3. Identification potential of DGGE

Recently, DGGE is being increasingly used for the analysis of LAB in food fermentation processes or products. For these applications, researchers are trying to optimize the DGGE method for direct identification of LAB without the use of additional identification techniques. In this regard, the use of **genus-specific primers** represents an important step, which generally results in a less complex DGGE banding pattern and a preselective amplification of the less abundant members of an ecosystem. Within the LAB, specific PCR primers targeting *Bifidobacterium* (Rigottier-gois *et al*., 2003) and *Lactobacillus/Pediococcus/Leuconostoc/ Weisella* (Walter *et al*., 2001; Heilig *et al*., 2002) have been designed. However, if too many different species are present, the banding pattern can remain very complex and identification of bands may still require sequencing. To this end, a part of the problem may be overcome by the **inclusion in each DGGE gel of an identification ladder** consisting of an artificial mixture of PCR amplicons from various pure cultures with known taxonomic identity. Band positions in the unknown sample lane can be visually compared with reference band positions in the identification ladder, leading to an identification. Meroth and co-workers (2003) used an identification ladder representing 13 *Lactobacillus* species for the identification of unknown *Lactobacillus* species in a sourdough fermentation process. These authors found this approach to produce better results compared to culture-dependent analysis using RAPD identification of purified LAB isolates. In an other study, two identification ladders containing 8 *Lactobacillus* species and 7 *Bifidobacterium* species, respectively, were included to analyse the microbial composition of probiotic products (Fasoli *et al*., 2003). Because of the limited complexity within the probiotic samples, no additional identification techniques were found necessary, although some probiotic LAB species could not be distinguished. It is evident that the composition of the identification ladder has to be chosen according to the expected species composition of the sample to be analysed, meaning that the availability of pre-existing information on the microbial diversity in the sample is crucial.

By means of changing different parameters, such as the electrophoresis conditions and denaturing gradient, DGGE can be adjusted in order to aid the optimal separation of bacterial species. Nevertheless, literature has not yet described DGGE as a completely independent identification technique for the analysis of bacterial communities.

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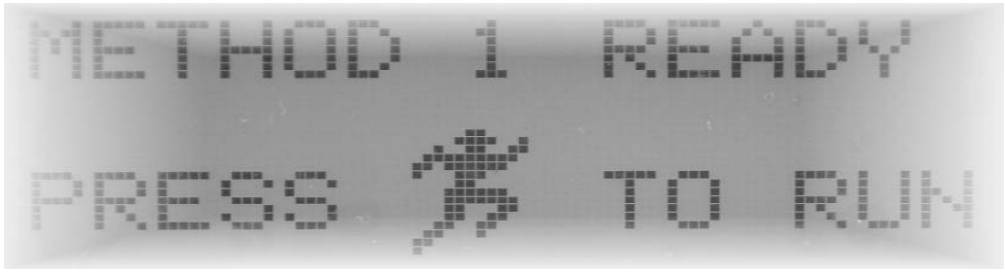
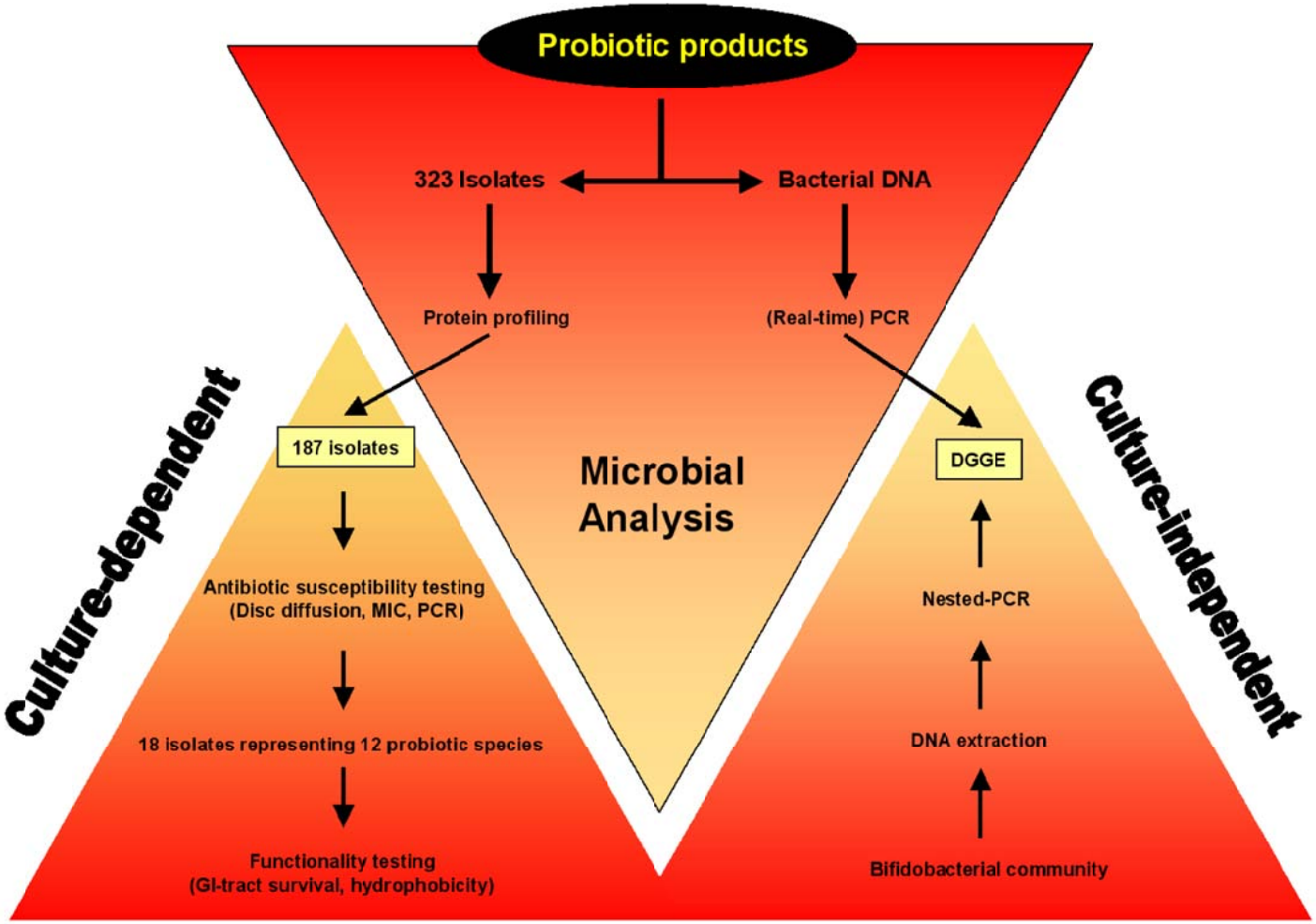
Part 2

Experimental Work




Probiotic products
323 Isolates
Bacterial DNA
Protein profiling
(Real-time) PCR
Culture-dependent
187 isolates
Antibiotic susceptibility testing
(Disc diffusion, MIC, PCR)
18 isolates representing 12 probiotic species
Functionality testing
(GI-tract survival, hydrophobicity)
Microbial
Analysis
DGGE
Nested-PCR
DNA extraction
Bifidobacterial community
Culture-independent

Part 2: Experimental work

Chapter 4: Culture-dependent microbial analysis of probiotics

4.1. Identification and antibiotic susceptibility of probiotic isolates

This study describes the isolation of bacteria from 55 European probiotic products, followed by their identification using protein profiling and the determination of their susceptibility to six antibiotics.

4.2. GI-tract survival capacity and hydrophobicity of probiotic isolates

A selection of 18 isolates representing 12 probiotic species was evaluated towards GI-tract survival capacity and hydrophobicity, as an indicator for potential adhesion.

Chapter 5: Culture-independent microbial analysis of probiotics

5.1. Development and optimization of Denaturing Gradient Gel Electrophoresis

The research in this section aimed the optimization of the DGGE technique in order to come to a culture-independent microbial analysis method for probiotic products on the qualitative level. A comparison with culture-dependent analysis was made using 10 probiotic products.

5.2. Nested-PCR DGGE for the microbial analysis of bifidobacterial communities

This study further optimizes DGGE as an identification method for the species-specific detection of bifidobacteria in various ecosystems, as demonstrated for fecal samples.

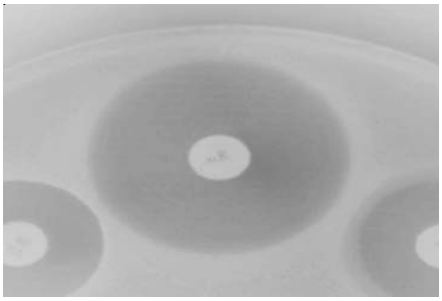
5.3. Real-time PCR DGGE

The coupling of real-time PCR to DGGE allows the complete culture-independent microbial analysis of probiotic products, both on a quantitative as well as qualitative level.

Chapter 4

Culture-dependent microbial analysis of probiotics

4.1. Identification and antibiotic susceptibility of probiotic isolates



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Summary

In the present study, a total of 55 European probiotic products were evaluated with regard to the identity and the antibiotic resistance of the bacterial isolates recovered from these products. Bacterial isolation from 30 dried food supplements and 25 dairy products, yielded a total of 268 bacterial isolates selected from several selective media. Counts of food supplements showed bacterial recovery in 19 (63%) of the dried food supplements ranging from 10^3 to 10^6 CFU/g, whereas all dairy products yielded growth in the range of 10^5 to 10^9 CFU/ml. After identification of the isolates using whole-cell protein profiling, mislabeling was noted in 47% of the food supplements and 40% of the dairy products. In six food supplements, *Enterococcus faecium* was isolated whereas only two of those products claim this species on their label. Using the disc diffusion method, antibiotic resistance among 187 isolates was detected against kanamycin (79% of the isolates), vancomycin (65%), tetracycline (26%), penicillinG (23%), erythromycin (16%) and chloramphenicol (11%). Overall, 68.4% of the isolates showed resistance against multiple antibiotics including intrinsic resistances. Initially, 38% of the isolated enterococci were classified as vancomycin resistant using the disc diffusion method, whereas additional phenotypical and PCR assays clearly showed that all *E. faecium* isolates were in fact vancomycin sensitive.

Keywords: Probiotics, Label Correctness, Identification, Antibiotic Susceptibility Testing

Introduction

The past five years have witnessed a strong expansion of the probiotic market and, in parallel, a rise in the number of research projects addressing fundamental and applied aspects of probiotics. New research technologies have supported earlier suggestions of health promoting properties of probiotic lactic acid bacteria (LAB) (reviewed by Naidu *et al.,* 1999) including stabilisation of the intestinal microflora by competition against pathogens (Gibson *et al.*, 1997), reduction of lactose intolerance (de Vrese *et al.*, 2001), prevention of antibiotic induced diarrhoea (Pochapin, 2000), prevention of colon cancer (Wollowski *et al.*, 2001), and stimulation of the immune system (Isolauri *et al.*, 2001). Bringing a probiotic to the market involves a step-wise process that needs to be carefully monitored in order to obtain a correctly labeled, functional, and safe product (Sanders and Huis in't Veld, 1999; Saarela *et al.*, 2000). If a product is not labeled correctly, safety and functionality cannot be guaranteed due to lack of documentation of the product components. However, as many manufacturers rely on the widely acknowledged but occasionally debated GRAS ('generally regarded as safe') status of lactobacilli and bifidobacteria (Salminen *et al.*, 1998), characterization of probiotic LAB strains with regard to taxonomic status, antibiotic resistance, and virulence may sometimes be neglected.

Microbial analyses of probiotic dairy products have demonstrated that the identity and the number of recovered species do not always correspond to the information stated on the product label (Reuter, 1997; Holzapfel *et al*, 1998; Hamilton-Miller *et al.*, 1999). However, it should be noted, that each of the cited studies were rather limited in number and type of products or were mainly restricted to national products. Various opinions exist as to whether it might be desirable that some probiotic strains show resistance to specific antibiotics that are for instance involved in antibiotic-induced diarrhea (Charteris *et al.*, 1998). On the other hand, the commercial introduction of probiotics containing antibiotic resistant strains may also have negative consequences, for example, when resistance is transferred to intestinal pathogens (Curragh and Colins, 1992).

In the current paper, an extensive study is presented to verify the label correctness of a range of European probiotic food supplements and dairy products, together with the antibiotic susceptibility testing of the product isolates. For each of these products, the label information was checked through taxonomic characterisation of the recoverable bacterial strains using whole-cell protein profiling. In addition, individual susceptibilities were determined for a selection of six antimicrobial agents.

Material and Methods

Bacterial isolation. A total of 55 probiotic products, collected in eight European countries, comprised 30 dried food supplements (**Table 1**) and 25 dairy products (**Table 2**). Dairy products were collected using a refrigerated box. None of the 55 products had exceeded their expiry date. All products were examined using a set of four isolation media under standardized cultivation conditions. For the isolation of *Lactobacillus* and *Lactococcus* strains, De Man Rogosa and Sharpe Agar (MRSA) medium (CM361, Oxoid, Basingstoke, UK) was used, whereas streptococci and enterococci were isolated on M17 medium (CM785, Oxoid) and on Kanamycine Aesculine Azide Agar Base (KAAAB)(CM591, Oxoid) respectively. For the isolation of bifidobacteria, Trans-Galacto-Oligosaccharides (TOS) medium (Matsuki *et al.*, 1999) was used with the following composition: 10g Trypticase Soy Broth (81-1768-0, Becton Dickinson, Sparks, USA), 1g Yeast Extract (L21, Oxoid), 3g KH_2PO_4 (1627, Vel, Leuven, Belgium), 4.8g K_2HPO_4 (1628, Vel), 3g $(NH_4)_2SO_4$ (1.01217.1000, Merck, Darmstadt, Germany), 0.2g $MgSO_4$-$7H_2O$ (1433, Vel), 0.5g L-cystein hydrochloride (C4820, Sigma, Bornem, Belgium), 15g Na-propionate (P1880, Sigma), 10g Transgalacto-OligoSaccharides (TOS, Honsha, Tokyo, Japan) and 15g agar (L11, Oxoid) dissolved in 1000 ml of distilled water. Products were sampled by preparing 10-fold dilutions of 100 µl of the dairy products or 100 mg of the food supplements in 10 ml pepton-physiological solution (PPS). A total of 50 µl of each dilution was plated in triplicate on all media, using the Whitley Automatic Spiral Plater (WASP^{TM}; Led Techno, Eksel, Belgium). All plates were incubated at 37°C under aerobic conditions, except for TOS plates that were incubated anaerobically (80% N_2, 10% H_2 and 10% CO_2) using an anaerobic chamber. After incubation for 48h, colony counts were performed and 3-5 colonies were picked based on different colony morphologies. Selected colonies were further purified on MRSA medium except that those recovered from TOS medium were cultured on Modified Columbia Agar (MCA) comprising 23g special pepton (L72, Oxoid), 1g soluble starch (1.01252.0250, Merck), 5g NaCl (1.06404.1000, Merck), 0.3g cystein-HCl-H_2O, 5g glucose (500520-887, Vel) and 15g agar dissolved in 1000 ml of distilled water. The latter medium was also used in a second screening round of products that claimed bifidobacteria on their labels, but did not produce any *Bifidobacterium* strain on the TOS medium during the first isolation round. Products that did not yield any isolates, were again screened, now using anaerobic and micro-aerophilic (3,5% CO_2, 5% O_2, 7,5% H_2, 84% N_2) cultivation conditions. In addition, these products were subjected to an enrichment step in MRS broth (CM359, Oxoid) using the same aerobic and anaerobic incubation conditions.

Identification of recovered isolates. Isolates were identified by Sodium Dodecyl Sulphate –Polyacrylamide Gelelectrophoresis (SDS-PAGE) analysis of whole-cell proteins, using standardized cultivation conditions for comparison with the available protein pattern database of lactic acid bacteria (Pot and Janssens, 1993). Extraction of cellular proteins was performed according to the method described by Pot *et al.* (1994) for Gram-positive bacteria. Extracts were separated using SDS-PAGE with a 5% total acrylamide stacking gel (12 mm long) and a 12% total acrylamide separation gel (126 mm long) . Gels were stained using Coomassie Blue R-250. The patterns were then densitometrically digitized using an LKB 2202 Ultrascan Laser Densitometer (LKB, Bromma, Sweden). Subsequently, these digital protein patterns were normalized using GelCompar software (Applied Maths, Sint-Martens-Latem, Belgium) so it became possible to identify the isolates by comparison of their protein patterns with the SDS-PAGE protein pattern database available at the laboratory. Upon repeated analyses, the inter-gel and intra-gel reproducibility was found to be 90.3% and 97.1%, respectively. Furthermore, the average correlation between reference patterns and the database standard was 95.3%, clearly surmounting the 94% limit for reliable identification.

Antibiotic susceptibility testing. At least one isolate per identified species recovered from a given product was included for antibiotic susceptibility testing, resulting in 187 isolates screened for possible resistance against a selection of six antibiotics, including kanamycin (30 µg), vancomycin (30 µg), erythromycin (10 µg), tetracycline (30 µg), chloramphenicol (30 µg) and penicillinG (10 µg) using a slightly modified version of the agar disc diffusion method (Kirby-Bauer, 1966). Strains were grown in MRS broth (Oxoid, CM 359) for 48h at 37°C. Following the preparation of a 10-fold dilution in PPS, freshly poured MRSA plates were equally inoculated with this dilution. Antibiotic discs (Oxoid, Basingstoke, UK) were placed on the inoculated plates using the Oxoid Disc Dispenser. Following a 24h incubation at 37°C, inhibition zones around the discs were measured using a digital callipers (Mauser, Switzerland). Results were interpreted according to the cut-off levels proposed by Charteris and co-workers (1998) with strains considered resistant if inhibition zone diameters were equal to or smaller than 19 mm for penicillinG, 14 mm for vancomycin and tetracycline, and 13 mm for kanamycin, chloramphenicol and erythromycin.

In addition to the agar disc-diffusion method, two other methods were used to confirm the presence of vancomycin resistance in enterococci. First, by growing the enterococci in a series of Trypticase Soy Broth (L21, Oxoid) Yeast Extract (211768, Becton Dickinson) (TSYE) tubes containing different concentrations of vancomycin or teicoplanin, the MIC (Minimal Inhibitory Concentration) for these antibiotics was determined according the protocol as

described by Arthur and Courvalin (1993). The combination of the MIC value obtained for both antibiotics is considered indicative for the preliminary classification as to what extent a strain is vancomycin resistance. In a second approach, the presumptive presence of vancomycin resistance was assessed, using a PCR protocol according to Dutka-Malen and co-workers, (1995) with primer pairs (A1, A2 and B1, B2) specific for the vancomycin resistance genes *vanA* and *vanB*.

Results

Bacterial isolation from probiotic products. Depending on the medium used, colony counts of the 25 investigated dairy products ranged from 10^5 CFU/ml to 10^9 CFU/ml. Among the 30 food supplements tested in this study, counts varied from 0 to 10^6 CFU/g. During a first isolation round, we were unable to isolate viable bacteria out of 12 (i.e. 40%) of the food supplements. These products were subjected to a second isolation round including an enrichment step in MRS broth and applying anaerobic as well as micro-aerophilic conditions. Only one of these 12 products displayed bacterial growth in MRSB but again not on MRS agar plates. At the end of the two isolation rounds, a total of 323 isolates were obtained. All isolated genera with exception of *Bifidobacterium* grew on all media used. However, it was noted that lactobacilli and enterococci grew best on MRSA and KAAAB, streptococci grew best on M17 and TOS, and bifidobacteria only grew on TOS.

Identification of recovered isolates. Identification results are presented in **Tables 1 and 2**. From a total of 323 isolates, 268 bacteria could be identified at the species level. The remaining 55 isolates were classified as yeasts after microscopical investigation or were lost during purification on MRSA medium. Only six products yielded all species indicated on the product label. However, when disregarding the presence of the yoghurt components *Streptococcus thermophilus* and *Lactobacillus delbrueckii* subsp*. bulgaricus*, this number of products rises to thirteen. In 19 products the isolated species were entirely different from those mentioned on the product label, even after a second isolation round using a new batch of the same products. In **Table 3**, a brief summary is given of the isolation and identification results. The most frequently recovered species among the food supplements was *Enterococcus faecium* followed by *Lactobacillus rhamnosus*. Of the 6 products in which *E. faecium* was found, only two actually claimed this species on their label. *Lactobacillus acidophilus,* which was claimed on the label of 22 food supplements, could only be isolated out of 2 of these products. Although all 13 food supplements claiming bifidobacteria were screened twice using two different media for the selective isolation of *Bifidobacterium* (TOS and MCA), only 3 of these 13 products produced a bifidobacterial strain. Among the 25 dairy products, *Lactobacillus acidophilus* was claimed, as well as isolated most frequently. As it was the case with the food supplements, only a poor retrieval of bifidobacteria was possible among the dairy products, despite the use of two different media. Only two out of 14 dairy products claiming bifidobacteria actually produced a *Bifidobacterium* strain during isolation.

Antibiotic susceptibility. Of the 268 identified isolates, 187 strains were subjected to antibiotic susceptibility testing using the agar disc diffusion method of which the results are presented in **Table 4.** It was found that 79% and 65% of the isolates were resistant to kanamycin and vancomycin, respectively. Furthermore, 23% and 21% of the isolates were grouped as resistant or intermediately resistant, respectively, to penicillin. Concerning the other antibiotics, the intermediate resistant fraction was never larger than 6,5%. It was also found that 38% of the isolated enterococci were vancomycin resistant according to the disc diffusion method. These resistant enterococci originated from 4 dried food supplements. However, when using the dilution method and a PCR assay for confirmation, none of the presumptively vancomycin resistant Enterococcus strains were found to be resistant against vancomycin (MIC < 2 µg/ml).

Table 1. Probiotic food supplements: Comparison of label claims with identification results of isolates from the products.

Product Name (type)	Producer (country)	Species claimed on the product label	Isolates from the product [1]
40+ Acidophilus (C)	Solgar Laboratories (The Netherlands)	*Lb. acidophilus, Lb. bulgaricus, B. bifidum, B. longum*	*P. acidilactici, Lb. plantarum, B. lactis*
ABCdophilus powder (P)	Solgar Laboratories (The Netherlands)	*B. bifidum, B. infantis, S. thermophilus*	No identification possible
Acidophilus bifidus (C)	Blackmores Ltd. (UK)	*Lb. acidophilus, B. bifidum*	No strains could be isolated
Acidophilus Plus (C)	Quest Vitamins (UK)	*Lb. acidophilus, Lb. rhamnosus, Lb. bifidum* [2]	*Lb. paracasei* ssp. *paracasei*
Acidophilus plus bifidus (C)	Kudos Vitamins & Herbals (UK)	*Lb. acidophilus, B. longum, B. infantis, B. casei* [2]	No strains could be isolated
Aciforce (P)	Biohorma (The Netherlands)	*Lb. acidophilus, Lc. lactis, E. faecium, B. bifidum*	*E. faecium, Lc. lactis* ssp. *lactis*
Bacilac (C)	THT (Belgium)	*Lb. acidophilus, Lb. rhamnosus*	No strains could be isolated
Bactisubtil (C)	Synthelabo Belgium (Belgium)	*Bacillus* IP5832	*Bacillus cereus*
Benefact (T)	Unknown	*Lb. bulgaricus*	No strains could be isolated
Beneflora (P)	ORTIS (Belgium)	*Lb. acidophilus, Lb. casei, B. longum, Lb. bulgaricus, S. thermophilus*	*Lb. acidophilus, B. longum, S. thermophilus*
Bifidus complex (C)	Biover (Belgium)	*Lb. acidophilus, Bifidobacterium, Saccharomyces cerevisiae*	*E. faecium*
Colon Clean Naturel (P)	Pharmafood (Belgium)	*Lb. acidophilus*	No strains could be isolated
Colon Clean Plus (P)	Pharmafood (Belgium)	*Lb. acidophilus*	No strains could be isolated
Colon Maintenance (C)	Holland & Barrett Ltd. (UK)	*Lb. acidophilus*	No strains could be isolated
Culturelle (C)	CAG Functional Foods (UK)	*Lb. GG*	*Lb. rhamnosus*
Diedam (P)	Almond Laboratorios (Spain)	*Lb. acidophilus, Lb. casei, Lb. bulgaricus, B. infantis, S. thermophilus*	No strains could be isolated

Table 1 (continued). Probiotic food supplements: Comparison of label claims with identification results of isolates from the products.

Product Name (type)	Producer (country)	Species claimed on the product label	Isolates from the product [1]
Effidigest (P)	Aca Pharma (Belgium)	N/A	*Lb. plantarum*
Lactéol (C)	Menarini Benelux (Belgium)	*Lb. acidophilus*	*Lb. rhamnosus*
Lactimum (C)	Biorès (Belgium)	*Lb. acidophilus, Lb. rhamnosus*	*E. faecium*
Lactobacillus Acidophilus (T)	Blackmores Ltd. (UK)	*Lb. acidophilus*	No strains could be isolated
Life Top Straw (PC)	BioGaia Biologics (Sweden)	*Lb. reuteri*	*Lb. reuteri*
Milk Free Acidophilus (C)	Holland & Barrett Ltd. (UK)	*Lb. acidophilus, Lb. bulgaricus, B. bifidum*	No strains could be isolated
Multi-billion dophilus (C)	Solgar Laboratories (The Netherlands)	*Lb. acidophilus, Lb. bulgaricus, S. thermophilus, B. bifidum*	*P. acidilactici*
Novaflora (C)	Pharmafood (Belgium)	*Lb. rhamnosus, Lb. lactis, E. faecium, Bifidobacterium*	*E. faecium*
Prévite acidophilus (C)	Unknown	*Lb. acidophilus*	*E. faecium*
Probiosan (T)	Nutrisan (Belgium)	*Lb. acidophilus, Lb. rhamnosus*	*Lb. crispatus, Lb. rhamnosus*
Proflora (C)	Chefaro (Belgium)	*Lb. acidophilus, Bifidobacterium, Lb. bulgaricus, S. thermophilus ,*	*Lb. acidophilus, B. lactis S. thermophilus*
Psyllium actif (P)	Biover (Belgium)	*Lb. acidophilus, Lb. bifidum* [2]	*E. faecium*
Superior Probiotics (T)	BioGaia Biologics (Sweden)	*Lb. reuteri*	*Lb. reuteri*
Vivaflora (T)	Laboratoires Super Diet (France)	*Lb. acidophilus, B. bifidum*	No strains could be isolated

Type of product: P = Powder, C = Capsule, T = Tablet, PC = Powder as Coating

(1) For *Bifidobacterium*, isolation results were obtained on TOS and MCA medium.

(2) indistinct or invalid name

Lb.. = *Lactobacillus, B.* = *Bifidobacterium, S.* = *Streptococcus, E.* = *Enterococcus, Lc.* = *Lactococcus, P.* = *Pediococcus*

Lb. bulgaricus = Corresponds to *Lactobacillus delbrueckii* ssp. *bulgaricus*

Table 2. Probiotic dairy products: Comparison of label claims with identification results of isolates from the products.

Product Name	Producer (country)	Species claimed on the product label	Isolates from the product [1]
Actimel	Danone (France)	*Lb. casei* Immunitas, living yoghurt cultures	*Lb. paracasei* ssp. *paracasei*
Actimel Orange	Danone (France)	*Lb. casei* Immunitas, living yoghurt cultures	*Lb. paracasei* ssp. *paracasei*
Almighurt	Almighurt (Germany)	Living yoghurt cultures	*Lb. bulgaricus, S. thermophilus*
B'A fruits	B'A (France)	*Bifidobacterium*[2]	*S. thermophilus*
B'A vanille	B'A (France)	*Bifidobacterium*[2]	*S. thermophilus*
Benecol	McNeil Consumer Nutritionals (UK)	*Bifidobacterium*[2]	*Lb. acidophilus, S. thermophilus*
BI'AC	TMA (Germany)	*Lb. acidophilus, Lb. casei*	*Lb. acidophilus, S. thermophilus, Lb. paracasei* ssp. *paracasei*
BIO abricot	Danone (France)	*Bifidobacterium,* living yoghurt cultures	*Lc. lactis* ssp. *lactis*
BIO framboise	Danone (France)	*Bifidobacterium,* living yoghurt cultures	*S. thermophilus, Lc. lactis* ssp. *lactis*
Biogarde halfvol Naturel	Strothmann (Germany)	*Lb. acidophilus, Bifidobacterium, S. thermophilus*	*Lb. acidophilus, S. thermophilus*
Biogarde plus (naturel)	Almhof (The Netherlands)	*Lb. acidophilus, Lb. casei, Bifidobacterium*	*Lb. acidophilus, S. thermophilus*
Biomild Drink	Mona (The Netherlands)	*Lb. acidophilus, B. longum, S. thermophilus*	*Lb. johnsonii, S. thermophilus, B. lactis*
Bio Snac'	Danone (France)	*Bifidobacterium,* living yoghurt cultures	*Lc. lactis* ssp. *lactis*
Fitness Quark	Onken (Germany)	*Lb. acidophilus* OCA5, *Bifidobacterium*	*Lb. johnsonii, S. thermophilus*
Fysiq	Mona (The Netherlands)	*Lb. acidophilus Gilliland,* living yoghurt cultures	*Lb. crispatus, S. thermophilus*
Gefilus	Valio (Finland)	*Lb. GG,* living yoghurt cultures	*Lb. rhamnosus*
Joghurt Mild Gartenfrucht	Bremerland (Germany)	*Lb. acidophilus* LA55, *Bifidobacterium* CB111	*Lb. johnsonii, B. lactis, S. thermophilus*

Table 2 (continued). Probiotic dairy products: Comparison of label claims with identification results of isolates from the products.

Product Name	Producer (country)	Species claimed on the product label	Isolates from the product [1]
Kinderyoghurt mild	J. Bauer KG (Germany)	*Lb. acidophilus, Lb. bifidus* [3]	*Lb. acidophilus, Lb. johnsonii, S. thermophilus*
Lactus Nature	Carrefour (France)	*Lb. casei* ssp. *rhamnosus,* living yoghurt cultures	*Lb. rhamnosus*
Lc1	Nestlé (Germany)	*Lb. johnsonii,* living yoghurt cultures	*Lb. johnsonii, S. thermophilus*
Natumild	Natuur Hoeve (The Netherlands)	*Lb. acidophilus, Lb. bifidus* [3]*, S. thermophilus*	*S. thermophilus*
Procult Drink	Alois Müller (Germany)	*B. longum* BB536¸ living yoghurt cultures	*Lb. acidophilus, S. thermophilus*
Vifit Drink	Mona (The Netherlands)	*Lb. casei GG* [3]*, Lb. acidophilus, B. bifidum*	*Lb. rhamnosus, Lb. acidophilus*
Weight Watchers Bifidus	Senoble (France)	*Bifidobacterium*[2]	*S. thermophilus*
Yakult	Yakult (The Netherlands)	*Lb. casei* Shirota	*Lb. paracasei* ssp. *paracasei*

All dairy products were fermented drinks or yoghurt based products.

(1) For *Bifidobacterium*, isolation results were obtained on TOS and MCA medium.

(2) Indicated on the product label as 'active bifidus'

(3) Indistinct or invalid name

Lb.. = *Lactobacillus, B.* = *Bifidobacterium, S.* = *Streptococcus, Lc.* = *Lactococcus. Lb. bulgaricus* = Corresponds to *Lactobacillus delbrueckii* ssp. *bulgaricus*

Table 3: Summary of isolation and identification results

Description	**Food supplements**	**Dairy Products**
Number of products	**30**	**25**
• from which no viable strains could be isolated	11 (37%)	0
• containing all claimed species	4/30 (13%)	2 (8%)
• containing other species than those claimed	9/19 (47%)	10 (40%)
• claiming more species than found	22/30 (73%)	16 (64%)
Most frequently claimed species	*L. acidophilus*	*L. acidophilus*
Most frequently isolated species	*E. faecium,*	*S. thermophilus,*
	L. rhamnosus	*L. acidophilus*

Table 4: Percent of isolates resistant against six tested antibiotics using the disc diffusion method.

Taxa (# strains tested)	**K (30 µg)**	**TE (30 µg)**	**E (10 µg)**	**P (10 µg)**	**C (30 µg)**	**VA (30 µg)**
Lactobacillus acidophilus (13)	100	8	8	0	8	69
Lactobacillus rhamnosus (24)	100	21	4	71	0	100
Lactobacillus casei (29)	100	7	10	17	10	100
Lactobacillus johnsonii (17)	100	0	6	0	0	76
Lactobacillus plantarum (6)	0	17	33	66	0	100
Lactobacillus reuteri (6)	100	100	0	100	33	100
Lb. delbrueckii subsp. *bulgaricus* (3)	67	0	0	0	0	67
Lactobacillus crispatus (6)	83	83	33	0	17	66
Streptococcus thermophilus (30)	60	3	0	3	10	40
Enterococcus faecium (29)	90	24	97	41	34	38
Lactococcus lactis subsp. *lactis* (8)	100	63	38	0	0	0
Pediococcus acidilactici (8)	0	38	0	25	38	100
Bifidobacterium longum (4)	100	0	0	0	0	0
Bifidobacterium lactis (4)	100	0	0	0	0	50
Total [1](187)	**79**	**26**	**16**	**23**	**11**	**65**

K = Kanamycin, TE = Tetracycline, E = Erythromycin, P = PenicillinG, C = Chloramphenicol, VA = Vancomycin
Lb. = *Lactobacillus*
[1] Total % of resistance calculated as the number of isolates (from a total of 187 isolates) resistant against the respective antibiotic.

Discussion

Considering the significant rise in the annual consumption of probiotic products worldwide, it is of paramount importance that these products are labeled correctly and that the probiotic strains are well-documented regarding safety and functionality (Sanders and Huis in't Veld, 1999). Hitherto, in Europe, there are no widely acknowledged regulations concerning the labeling issues and claims that can be made by the manufacturers of functional foods (Berner *et al.*, 1998; Przyrembel, 2001). Our findings clearly indicate the need for such regulations. Although freeze- or spray-dried cultures should yield probiotic products with a high concentration of viable bacteria, counts were subtantially lower among the 30 food supplements compared to the 25 dairy products. Possibly, higher isolation numbers could have been obtained when positive food supplements were processed using anaerobic isolation conditions. However, it can be speculated that the significant difference in relative numbers between the two main types of products (i.e. dried food supplements and dairy products) will not be affected by incubation under aerobic or anaerobic conditions. Therefore, it is possible that a number of the investigated food supplements may comprise a bacterial concentration below the minimum value required for any probiotic strain to affect the gastro-intestinal tract and thus to be able to promote a significant health effect. Using our protocol, a total of 11 food supplements (37%) did not yield any viable bacteria on the four isolation media. It can be speculated that the absence of living bacteria in a dried food supplement is due to low efficiencies of the drying and capsulation process, the possibility that some of these food supplements were sterilised for safety reasons, or because of a too long shelf-life period. The products investigated had not yet reached the expiry date at the moment of the isolation procedure. A product is designed to be of good quality upto the date of expiry, therefore the moment within its shelf life at which the analysis was performed, should have no reflection on the results obtained. Although some immunological activities have been assigned to dead bacteria (Wagner *et al*., 2000), many health promoting properties, e.g. competitive exclusion of pathogens, nutrient supplementation for the host, and anti-tumor effects can only be exerted by living bacteria (Ouwehand and Salminen, 1998). Given the present findings, it is therefore more likely for dairy products to exert these probiotic properties than it is for dried food supplements. No data on the viability and numbers of bacteria in the product at the time of consumption could be deduced from the product labels.

Identification of 268 isolates using protein profiling revealed that *Enterococcus faecium* was the most frequently recovered species out of the food supplements. This taxon was found in 6 out of the 19 food supplements (32%) containing living bacteria. With the exception of one product, *E. faecium* was the only species isolated out of these food supplements (**Table 1**). Because of the high isolation numbers (10^4 – 10^5 CFU/g), it is unlikely that *E. faecium* entered the production process via contamination. The second most frequently recovered species in food supplements was *Lactobacillus rhamnosus* followed by *Lactobacillus acidophilus* which was claimed to be present in 22/30 (73%) products but was only found twice (**Table 1**). Although a poorer growth was observed for *L. acidophilus* on MRSA medium compared to *Lb. rhamnosus, Lb. casei* and *E. faecium*, the relatively low recovery rate of the former species cannot be clearly explained. Likewise, the poor retrieval of bifidobacteria could not readily be explained because isolation results were comparable after testing various isolation parameters, e.g. atmosphere, temperature and duration of incubation (data not shown). More likely, it is possible that the nutritional content of the TOS and MCA medium used in this study did not meet the specific growth requirements of a number of probiotic bifidobacterial strains. Therefore, it can be speculated that more products claiming bifidobacteria may have produced these organisms during isolation, when a series of well-defined strain-specific media were used. The need for broad-spectrum isolation media for bifidobacteria is clearly demonstrated by this study and has also been suggested by Roy (2001). Among the tested food supplements, a total of 9 products contained species other than those stated on the product label. This mislabeling has also been reported previously by Hoa *et al.* (2000) for *Bacillus* containing food supplements and by Hamilton-Miller *et al.* (1999) for twenty out of 29 tested food supplements.

Since *Streptococcus thermophilus* and *Lb. delbrueckii* subsp. *bulgaricus* are the main composites of yoghurt, it could be expected that these two species were among the most frequently isolated ones from the dairy products. However, *Lb. delbrueckii* subsp. *bulgaricus* was only found once possibly because this species is rapidly overgrown by other lactobacilli in the dairy products. The fact that *Lb. acidophilus* was more easily isolated from dairy products than from food supplements, could be related to (1) the supporting matrix of the product in which the strains have to survive for the complete shelf-life, (2) the ambient temperature at which the different products are usually maintained, (3) the total shelf-life (average of 30 days for dairy products, average of 24 months for food supplements) or (4) to the individual strain differences with respect to survival in the stationary phase at the given temperature. Fourteen dairy products also claim to contain bifidobacteria, whereas in only two instances, *Bifidobacterium lactis* was recovered instead of the claimed *Bf. longum*. As outlined above,

the low recovery of bifidobacteria might be due to the lack of optimal isolation media for specific *Bifidobacterium* strains. Although to a lesser extent than with food supplements, our results suggest that also quite a number of dairy products suffer from mislabeling, which underscores similar findings of other workers (Reuter, 1997; Holzapfel *et al*., 1998; Hamilton-Miller *et al.*, 1999).

Using the disc diffusion method, high frequencies of resistance were detected for kanamycin (79%) and vancomycin (65%). Most of the kanamycin resistant isolates belonged to the genera *Lactobacillus* and *Enterococcus*. The latter genus is intrinsically resistant against kanamycin (Franz *et al.*, 1999), but the finding that 81% of the isolated lactobacilli were also resistant against kanamycin somewhat counteracts the specificity of the *Enterococcus*-specific KAAAB medium. Likewise, the relatively high percentage of vancomycin resistance observed amongst the entire collection of isolates is due to the fact that the majority of the lactobacilli are intrinsically resistant to this glycopeptide (Nelson, 1999). Noteworthy, intraspecific variations were found among the *Lb. johnsonii* and *Lb. acidophilus* isolates, which is in agreement with previous observations of Charteris and co-workers (1998). Strikingly, 38% of the *Enterococcus faecium* isolates also showed to be resistant against vancomycin according to the disc diffusion method. However, these findings could not be confirmed by the dilution method (Arthur and Courvalin, 1993) or by a PCR-based *van* gene detection assay (Dutka-Malen *et al*., 1995). Collectively, these findings demonstrate that all enterococci isolated from probiotic products were susceptible to vancomycin, which again highlights the limited reliability of the disc diffusion method to determine the occurrence of vancomycin resistance with enterococci (Swenson *et al*., 1989). The high frequencies of vancomycin resistance found among other lactic acid bacterial genera does not pose a problem as this type of vancomycin resistance is different from the inducible, transferable mechanism observed in enterococci (Salminen *et al.* 1998; Klein *et al.*, 2000). The lactobacilli in the present study comprised strains resistant to tetracycline (29.5%) chloramphenicol (8.5%), and erythromycin (12%) and overall, more than 68% of our isolates exhibited resistance to two or more antibiotics (data not shown), including some intrinsic resistances. With regard to general concerns on biosafety of probiotics, further research should focus on the location and potential transferability of these antibiotic resistance determinants.

In conclusion, it can be stated that quite a number of dried food supplements and - to a lesser extent - dairy products are incorrectly or inadequately labeled with regard to the correct identity of the incorporated probiotic strains. Despite earlier reports concerning mislabeling of probiotic products (Reuter, 1997; Holzapfel *et al*., 1998, Hamilton-Miller *et al.*, 1999), the new data indicate that this situation has not significantly improved. Although specific antibiotic resistance traits among probiotic strains may be desirable (Charteris *et al*., 1998), the finding of tetracycline, chloramphenicol and erythromycin resistance among the investigated probiotic isolates indicates that continuous attention should be paid to the selection of probiotic strains free of transferable antibiotic resistance. It is of paramount importance that at a time when consumers become more aware of the importance of good nutrition and health, probiotic products designed especially for their health promoting purposes are safe and well-documented in order to provide consumers with the full benefits of the remarkable aspects of probiotics.

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4.2 GI-tract survival capacity and hydrophobicity of probiotic isolates

R. Temmerman[1], D. Goudercourt[2], S. Poiret[2] and B. Pot[2].

[1] Lab of Microbiology, Ghent University, Belgium.

[2] Bacteriology of Ecosystems, Institut Pasteur de Lille, France.

Introduction

Numerous studies have already demonstrated multiple beneficial features of probiotic bacteria (Naidu *et al*., 1999). Although the discussion whether probiotic strains should be alive or not in order to exert beneficial effects is ongoing, it is evident that live bacteria will certainly facilitate health benefits impossible to result from dead bacterial cells. However, this implies that these bacteria should also reach the site of action, i.e. large intestine, alive. To survive passage through the stomach and small intestine, probiotic strains must tolerate the acidic and protease-rich conditions of the stomach, and survive and grow in the presence of bile acids. Furthermore, acid tolerance is also important for the probiotics' survival in food (Lee and Salminen, 1995). Yoghurts and fermented milks remain the dominant food vehicle for probiotics; both provide a relatively low-pH environment in which the probiotic bacteria must survive. Together with passage through the stomach, acid tolerance is thus one of the first properties screened for when selecting probiotic strains; simple *in vitro* tests displaying representative results (Tuomola *et al*., 2001). Similar *in vitro* assays examining the inhibitory effect of bile acids on the growth of probiotic strains are also relatively simple to perform, although, quantitative extrapolation to probiotic performance *in vivo* is difficult. Intraspecies variation in the ability to grow in the presence of bile is often observed between potential probiotic strains (Dunne *et al*., 2001), and *in vitro* tests can be used to select the best strains on a relative basis. Once a probiotic strain has survived passage to the large intestine, an important determinant for its functionality is the potential of the strain to adhere to mucus and/ or epithelial cells. In several studies, adhesion was related to a shortening of duration of diarrhoea, immunogenic effects, competitive exclusion and other health effects (Salminen *et al*., 1998). However, adhesion characterization through the use of cell lines may be a rather laborious procedure and requires expertise. Because the electron donor and acceptor properties, as well as the hydrophobicity of the bacterial cell wall have been linked to the adhesion potential of the strain (Wadström *et al*., 1987, Briandet *et al*., 1999), these more easy assessable characteristics represent a good alternative.

The following study applies a series of *in vitro* tests to screen 18 probiotic isolates, representing 12 species, for their degree of resistance against pepsine, low pH and pancreatine, as well as for their growth performance in the presence of bile salts. Furthermore, using the MATS test (Boonaert *et al*., 2001), hydrophobicity and the electron donor and acceptor properties of the isolates were determined, as a possible indication for their adhesive potential (Girardin *et al*., 1999). These *in vitro* tests for selection of acid- and bile tolerant strains can readily be applied to select probiotic strains with the highest chances towards functionality.

Material and Methods

Strain collection. From the previous study by Temmerman and co-workers (2003) eighteen isolates (**Table 1**) representing twelve different probiotic species were selected on the basis of their identification, total counts in the source product and antibiotic susceptibility profile.

Pepsin resistance. In combination with a low pH, the presence of the enzyme pepsin in the stomach constitutes the first human antimicrobial barrier encountered by a probiotic bacterium. The potential of the eighteen isolates towards passage through the stomach was evaluated by determining the percentage of bacteria that survives inoculation in an acidic pepsin containing solution. Bacterial strains were grown in 10 ml of MRS or M17 broth (Difco) at 37°C during 24h after which the cell suspension was centrifuged for 5 min at 4000 rpm. The pellet was washed 3 times in PBS buffer (pH7) and finally dissolved in 1 ml of PBS. Of this cell suspension 200 µl was added to a series of 4 tubes containing 1 ml of filtered pepsin (Sigma) solution at pH 2 and 300 µl of NaCl. Immediately after inoculation (T0), a 10-fold dilution series of 100µl cell suspension from tube 1 was made in Ringer solution (Merck) and subsequently plated on MRSA or M17 agar (Difco) for incubation at 37°C for 24h. The same procedure for enumeration was performed for the other 3 tubes after T0 + 20 min, T0 + 40 min and T0 + 60 min, respectively.

Pancreatin resistance. The resistance of the 18 isolates against pancreatin was assessed in a similar approach as for pepsin. After washing, 200 µl cell suspension was added to a series of 5 tubes containing 1 ml of filtered pancreatin (Sigma) solution at pH 8 and 300 µl of NaCl. Again, dilution series for enumeration on MRSA and M17 were prepared at T0, T0 + 20 min, T0 + 40 min, T0 + 60 min and T0 + 120 min.

Bile salt resistance. The resistance of the 18 probiotic isolates to bile salts was determined by comparing their growth capacity in the presence and absence of a mixture of bile salts as described by Gilliland and co-workers (1984). Bacterial strains were grown in 10 ml of MRS or M17 broth at 37°C during 24h after which two 30 ml tubes per strain of MRS or M17 broth were inoculated with the culture until an initial OD (540 nm) between 0.05 and 0.1 was obtained. In one tube, 750 µl of a filter sterilised bile salt solution (Sigma) was added to

obtain a final concentration of 0.3% of bile salts. Both tubes were incubated at 37°C and OD was measured frequently until both solutions reached an OD value of 0.3. The difference in time for both solutions to obtain this OD value is a measure for the sensitivity of the bacterium to bile salts (Chateau *et al*., 1994).

Hydrophobicity test. As an indication for adhesion potential, the hydrophobicity (or polarity) of the 18 isolates was determined by means of using 5 solvents {decane, hexadecane, ethylacetate, xylene (Sigma) and chloroform (Merck)}. The principle of this MATS (Microbial Adhesion To Solvents) test (Boonaert *et al*., 2001) is based on the partition of the bacteria between two non-intermixible liquid phases in regard to the 5 solvents. Bacterial strains were grown overnight in 10 ml of MRS or M17 broth at 37°C, after which the cultures were centrifuged for 10 min at 3500 rpm at 4°C. The pellet was washed twice in 5 ml of PBS, by cetrifugation for 10 min at 3500 rpm, after which the pellet was again dissolved in 5 ml of PBS buffer. The Optical Density (OD) measured at 540 nm (Beckman DU640B spectrophotometer (Beckman, USA)) was set at 0.6 **(OD = A0)** by means of dilluting the cell suspension using PBS. In triplicate, 3 ml of cell suspension and 250 µl of solvent were joined in a glass tube, followed by 1 min of vortexing. The mixture was set to rest during 30 min, after which the OD of 1 ml of the watery phase was measured **(OD = A)**. The percentage of bacteria adhering to the solvent (hydrophobicity nature) was calculated as: **(1-A/A0)x100.**

Results and Discussion

Besides health promoting effects, the functional aspect of probiotic strains also involves their survival capacity to reach the large intestine, as well as their adhesion potential to mucus or enterocytes. Based on the isolation, identification and antibiotic susceptibility results from a previous study (Temmerman *et al*., 2003), 18 isolates representing 12 different probiotic species were selected for the purpose of this study. Using a number of *in vitro* tests, all of these strains were assessed towards their resistance against low pH, pepsin, pancreatin and bile salts, as well as for their hydrophobic nature as a possible indication of adhesion potential. Such *in vitro* studies can be employed in the selection of LAB for use in probiotic functional foods and nutraceutical preparations (Charteris *et al*., 1998).

About 2.5 l of gastric juice is secreted each day having a pH of approximately 2.0 and a salt content of no less than 0.5% w/v (Hill, 1990). In contrast, about 0.7 l of pancreatic juice is secreted into the proximal small intestine each day having a pH of about 8.0 with a similar salt content (Keele and Neil, 1965). These secretions present a pH and enzymatic barrier to the survival of ingested microorganisms during digestion and act in concert with bile and peristalsis to ensure that the resting small intestine is only heavily colonized in conditions of stasis (Charteris *et al*., 1998). Nevertheless, probiotic species will need to survive this barrier in adequate amounts in order to exert their effects in the large intestine. The potential of the eighteen isolates towards passage through the stomach was evaluated by determining the percentage of bacteria that survive a certain inoculation time (i.e. 0, 20, 40 and 60 min) in an acidic pepsin containing solution (**Table 1**). Even after 60 min, the reduction in viability of *Lactobacillus crispatus*, *L. reuteri* and both *L. johnsonii* isolates was very low, as previously described by du Toit and co-workers (1998). *Lactobacillus acidophilus* and one isolate of both the *L. casei* and *L. plantarum* isolates survived well up to 40 min and can thus be considered tolerant to gastric juices. The remaining isolates showed considerably less survival capacity, although all of the probiotic isolates scored considerably better than the two starter cultures *L. delbrueckii* subsp*. bulgaricus and S. thermophilus* being very sensitive to low pH, confirming earlier observations (Conway *et al*., 1987). These results clearly indicate the difference between selected probiotic strains and starter cultures, which are sometimes considered to be probiotic as well. Research by Charteris and colleagues (1998) has determined that for certain probiotic strainspassage through the stomach can be enhanced by the presence of milk proteins, hereby suggesting that milk-based products constitute an important carrier of probiotic strains.

After the stomach, probiotic bacteria end up in the small intestine where the presence of pancreatin together with bile salts and other enzymes form another serious barrier, although the more neutral pH favours survival of the bacteria. Except for both *L. casei* isolates (R15649 in particular), all tested isolates showed high resistance against the enzym pancreatin. Also, both starter cultures survived inoculation with pancreatin completely (**Table 1**). The observation that for a number of strains the percentage of survival remounts after a certain time of incubation, might be due to the fact that most strains are not only resistant to pancreatin, but regain their ability to grow as well. The classification of the isolates in their resistance against bile acids was performed according to Chateau *et al*., 1994: if the difference in time between a bacterial suspension with or without bile acids to reach OD 0.3 was below 15 minutes, the isolate was considered to be resistant to bile acids. Between 15 and 40 min, the strain is tolerant and when the delay is longer than 60 minutes the isolate is sensitive for the presence of bile acids (between 40 and 60 minutes we talk about weak tolerance). In **Table 1** it can be seen that 6 of the isolates are resistant to bile acids, 7 are tolerant and 5 are sensitive. As expected, the starter cultures were sensitive, meaning that in combination with their low potential for gastric survival, these strains are unlikely to reach the large intestine alive. The high resistance for pancreatin, as well as the variable results among different strains for resistance to bile acids have also been reported by Charteris and co-workers (1998) among 15 *Lactobacillus* and *Bifidobacterium* strains. Our results show that mainly *L. crispatus, L. plantarum, L. reuteri and L. johnsonii* are suitable candidates for functional food product development with regard to their GI-tract survival capacity, although the strain-dependent nature of the results has to be taken into account.

Wadström and colleagues (1987) suggested that the hydrophobic or hydrophyllic nature of the bacterial cell surface may be a determinant for the adhesion potential of the strain, which is an important criterium for colonization (Finlay and Falkow, 1997). Also the electron donor and acceptor properties may take part in bacterial adhesion (Briandet *et al*., 1999). *In vitro* tests have been designed and applied to address this issue (Boonaert *et al*., 2001), although a clear correlation between hydrophobicity and adhesion of a probiotic strain has not yet been found (Ouwehand *et al*., 1999). In this study, partitioning of the isolates into five solvents (chloroform, decane, hexadecane, xylene and ethylacetate) was determined. An affinity to chloroform, being an acidic solvent, reflects the reducing (alcalic) nature of the bacteria. Affinity to ethylacetate, being an alcalic solvent, reflects the oxydising (acidic) nature of the bacteria. Furthermore, affinity towards the apolar solvents (decane, hexadecane and xylene) demonstrates the hydrophobic nature of the bacteria; a high hydrophobicity being linked to

glycoproteins on the bacterial surface, and a low hydrophobicity being linked to the presence of polysaccharides on the bacterial surface. The results presented in **Table 2** show that 75% of the strains demonstrate a clear affinity to chloroform, and that the remaining 25% is of a more neutral character. Concerning the hydrophobic nature of the bacteria, approximately a third of the bacteria displayed high, neutral and low affinity towards the three solvents. Although the correlation between these results and the actual adhesion potential of the strains was not determined, these results again demonstrate a high variability among the strains tested.

Although the relevance of some of the performed *in vitro* tests towards the actual *in vivo* situation is not always clear, these kind of tests allow a fast indication about the main characteristics of a certain probiotic strain. The large strain-dependent results for stomach survival and hydrophobicity demonstrate that different strains may display substantial differences in functionality. As a result, these *in vitro* as well as further *in vivo* tests are of great value in selecting the most functional probiotic strain.

Table 1. Resistance of probiotic isolates against pepsin, pancreatin and bile acids.

Isolate	Strain nr.	Pepsin survival (%)				Pancreatin survival (%)					Bile resistance
		5min	20min	40min	60min	5min	20min	40min	60min	120min	
L. plantarum	R10692	76.6	44.3	36	0	89.2	68	77	100	78	Resistant
L. plantarum	R10686	116	25	4.6	2.9	100	100	99	100	100	Resistant
Lc. lactis subsp. *lactis*	R15646	27	21	0	0	100	100	100	69.4	100	Resistant
Lc. lactis subsp. *lactis*	R15654	73	0	0	0	100	100	100	100	100	Resistant
S. thermophilus	R15652	2.2	0	0	0	98.9	81.5	78.2	85.7	65.8	Sensitive
L. crispatus	R15657	89	90	60	42	29	100	100	100	100	Tolerant
L. rhamnosus	R15653	10.6	0	0	0	100	100	100	100	100	Sensitive
L. rhamnosus	R15655	19.7	5	1.8	0	100	98.6	100	100	100	Sensitive
L. acidophilus	R15650	53	26	21	19	87	100	100	34	100	Sensitive
L. casei	R15648	60.9	45.1	34.6	16.9	60	75	60	62	47.6	Tolerant
L. casei	R15649	3.6	9.3	0.3	0	42	28.4	14.1	13.3	15	Tolerant
L. reuteri	R15647	100	106	127	59	100	81	100	100	92	Resistant
L. johnsonii	R15656	100	92.1	98	88.5	100	100	100	100	100	Tolerant
L. johnsonii	R15659	85.3	79.8	86.3	78.5	100	100	86.5	93.3	94.9	Tolerant
L. bulgaricus	R15661	0	0	0	0	100	100	56	78	100	Sensitive
E. faecium	R11413	100	95.2	0.27	0	100	100	100	100	100	Resistant

Table 2. Hydrophobicity testing of probiotic isolates as an indication for adhesion capacity.

Isolate	**Strain nr.**	**Hydrophobicity testing**				
		Decane	**Hexadecane**	**Xylene**	**Ethylacetate**	**Chloroform**
L. plantarum	R10692	8.6	11.3	38.1	5.2	43.6
L. plantarum	R10686	32.1	31.3	45.6	9.6	56.4
Lc. lactis subsp. *lactis*	R15646	7.3	5.1	2.7	11.4	17.9
Lc. lactis subsp. *lactis*	R15654	24.4	6.4	20.9	2.6	42.9
S. thermophilus	R15652	8.1	2.2	21.9	7.9	28.6
L. crispatus	R15657	45.3	27.8	82.4	9.9	93.3
L. rhamnosus	R15653	3.9	1.6	5	11.5	36.9
L. rhamnosus	R15655	2.4	0.9	0.4	5.8	9.8
L. acidophilus	R15650	7.4	7.1	28.2	2.8	26.9
L. casei	R15648	17.2	12.4	43.9	2.6	37.7
L. casei	R15649	13	3.2	30.8	24.9	41.3
L. reuteri	R15647	18.3	11.3	14.7	4.8	25.4
L. johnsonii	R15656	39.4	30.4	44.6	4.2	65.9
L. johnsonii	R15659	43.8	41	47.4	2.4	65.6
L. bulgaricus	R15661	5.6	5.5	7.1	7.3	18.4
E. faecium	R11413	3.28	2.49	30.75	2.47	2.48

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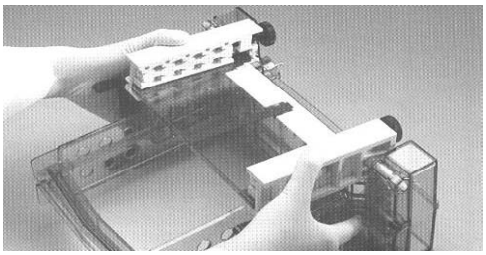
Chapter 5

Culture-independent microbial analysis of probiotics

5.1. Development and optimization of Denaturing Gradient Gel Electrophoresis (DGGE)



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Summary

In order to obtain functional and safe probiotic products for human consumption, a fast and reliable quality control of these products is crucial. Currently, analysis of most probiotics is still based on culture-dependent methods involving the use of specific isolation media and the identification of a limited number of isolates, which renders this approach relatively insensitive, laborious and time-consuming. In the current study, a collection of ten probiotic products including four dairy products, one fruit drink and five freeze-dried products were subjected to microbial analysis using a culture-independent approach in comparison with conventional culture-dependent analysis. The culture-independent approach involved extraction of total bacterial DNA directly from the product, PCR amplification of the V3 region of the 16S rDNA, and separation of the amplicons on a Denaturing Gradient Gel Electrophoresis (DGGE) gel. Digital capturing and processing of DGGE band patterns, allowed direct identification of the amplicons on the species level. This whole culture-independent approach can be performed in less than 30 hours. In comparison with culture-dependent analysis, the DGGE approach was found to have a much higher sensitivity for the detection of microbial strains in probiotic products in a fast, reliable and reproducible manner. Unfortunately, as reported in earlier studies using the culture-dependent approach, a rather high percentage of probiotic products suffered from an incorrect label and yielded low bacterial counts, impairing beneficial probiotic effects.

Introduction

According to the Food and Agriculture Organization, a probiotic is a live microorganism which when administered in adequate amounts confers a health benefit on the host, and due to the increasing importance of health during the past decade, a proportional expansion of the probiotic product market can be witnessed (Stanton *et al*., 2001). Although probiotics were originally based on fermented dairy products, at present, numerous probiotic food supplements are now also commercially available as tablets, powders or capsules. Bringing a functional, safe and correctly labelled probiotic product to the market demands a careful monitoring of the whole production process (Saarela *et al*., 2000). Previous analyses of probiotic products have demonstrated that the identity and number of recovered microbial species do not always correlate with the information stated on the product labels (Hughes *et al*., 1990, Hamilton-Miller *et al*., 1996, 1999; Yeung *et al*., 2002, Temmerman *et al*., 2003). These and other studies mainly relied on the use of culture media to isolate the bacteria present in the probiotic product, after which a selection of purified isolates is identified using 16S rDNA sequencing (Holzapfel *et al*., 2001; Yeung *et al*., 2002), Restriction Fragment Length Polymorphism analysis (Zhung *et al*., 1998) or protein profiling (Temmerman *et al*., 2003).

However, because these cultivation-dependent approaches have proven limitations in terms of recovery rate and reproducibility, the set of recovered isolates may not always truly reflect the microbial composition of the product (Ampe *et al*., 1999; Ercolini *et al*., 2001; Roy, 2001). Moreover, a more comprehensive insight in the production process and the survival capacity of the introduced strains requires analysis of both viable and non-viable bacteria. In practice, the need to identify product isolates (mostly lactic acid bacteria) at least to the species level makes the cultivation-based procedure rather time-consuming.

In the current study, a cultivation-independent method is presented to detect and identify bacteria in probiotic products in a fast and reliable manner. Essentially, the protocol comprises three steps: (i) extraction of bacterial DNA from the probiotic product, (ii) PCR amplification of a specific part of the 16S rDNA gene, and (iii) electrophoresis of 16S rDNA amplicons using Denaturing Gradient Gel Electrophoresis (DGGE). At present, DGGE analysis is one of the

most suitable and widely used methods to study complex bacterial communities in various environments (Muyzer *et al*., 1998). In comparison to highly complex ecosystems such as the animal or human intestinal tract, a probiotic product can be considered as a rather simple microbial community and therefore the DGGE method should allow the qualitative analysis of any probiotic sample. The DGGE based approach presented in this paper, can also be used as a culture-independent identification method. In less than 30 hours, a given probiotic product can be analyzed to verify the species composition stated on its label. In order to validate this DGGE approach, the same products were also screened using conventional cultivation on selective isolation media, followed by identification of the recovered isolates using SDS-PAGE protein profiling.

Material and Methods

Probiotic products. A total of 10 commercially available probiotic products were analysed in this study, including 5 freeze-dried products, 4 dairy products and 1 fruit drink (**Table 1**). Besides the type of product, the choice was also based on the number of different bacterial groups claimed on the product label. As shown in **Table 1**, the investigated products contained one to four different bacterial species.

Bacterial strains. All products were examined using a set of four isolation media under standardized cultivation conditions. For the isolation of *Lactobacillus* and *Lactococcus* strains, De Man Rogosa and Sharpe Agar (MRSA) medium (CM361, Oxoid, Basingstoke, UK) was used, whereas streptococci and enterococci were isolated on M17 medium (CM785, Oxoid) and on Kanamycine Aesculine Azide Agar Base (KAAAB)(CM591, Oxoid) respectively. For the isolation of bifidobacteria, Trans-Galacto-Oligosaccharides (TOS) medium was used with the following composition : 10g Trypticase Soy Broth (81-1768-0, Becton Dickinson, Sparks, USA), 1g Yeast Extract (L21, Oxoid), 3g KH_2PO_4 (1627, Vel, Leuven, Belgium), 4.8g K_2HPO_4 (1628, Vel), 3g $(NH_4)_2SO_4$ (1.01217.1000, Merck, Darmstadt, Germany), 0.2g $MgSO_4$-$7H_2O$ (1433, Vel), 0.5g L-cystein hydrochloride (C4820, Sigma, Bornem, Belgium), 15g Na-propionate (P1880, Sigma), 10g Transgalacto-OligoSaccharides (TOS, Honsha, Tokyo, Japan) and 15g agar (L11, Oxoid) dissolved in 1000 ml of distilled water. Identification of the isolates was performed using SDS-PAGE separation of extracted cellular proteins as described previously (Temmerman *et al*., 2003). In order to verify the reliability of the DNA extraction protocol for probiotic products and to verify the identification potential of DGGE, cell suspensions of type strains were made, with the aim to simulate the species composition of the products. These cell suspensions were prepared by harvesting half a loop of cells with a sterile öse from a freshly grown pure culture on MRSA medium (Oxoid, CM 361), and homogeneously suspending these cells in 10 ml of Peptone Physiological Solution (PPS) [0.1% (w/v) peptone (Oxoid, L37) and 0.85% (w/v) NaCl].

DNA extraction. Extraction of total bacterial DNA was based on the method described by Pitcher and co-workers (1989) with slight modifications depending on the type of starting material. For dairy products, 1 ml of product was centrifuged for 10 min at 13.000 rpm (Eppendorf Centrifuge 5804R, Hamburg, Germany), followed by removal of the supernatant

and resuspension of the pellet in 1 ml of Tris EDTA (TE-) buffer. Because of the large fruit components in the fruit drink, 50 ml of the drink was centrifuged for 2 min at 1000 rpm, after which 1 ml of the top liquid was taken and centrifuged for 10 min at 13.000 rpm. After removal of the supernatant, the remaining pellet was dissolved in 1 ml of TE-buffer. In case of the capsule type products, the content of one capsule, corresponding to approximately 100mg, was dissolved in 10 ml of sterile PPS and softly shaken until a homogeneous suspension was obtained. One ml of this suspension was transfered to an eppendorf tube and submitted to a centrifugation step of 10 min at 13.000 rpm, after which the supernatant was removed and the remaining pellet was suspended in 1 ml of TE buffer. This procedure was also applied for freeze-dried powders, of which 100 mg was weighted and suspended in 10 ml PPS. For freeze-dried tablets, one tablet was crushed in a sterile mortar and the obtained powder was dissolved in 10 ml of PPS and again homogenised. Of this suspension, 1 ml was centrifuged for 10 min at 13.000 rpm and the remaining pellet was dissolved in 1 ml of TE buffer. All cell suspensions in TE buffer were centrifuged for 5 min at 13.000 rpm. The supernatant was removed and 150 µl of a lysozyme solution [5 mg of lysozyme (28262, Serva, Heidelberg, Germany) in 150 µl of TE buffer] was added, followed by an incubation at 37°C during 40 min. In case of DNA extraction from pure cultures, only this lysozyme step was added to the protocol by Pitcher and co-workers (1989). Obtained DNA was dissolved in 200 µl TE buffer overnight after which an RNA digesting step was performed by adding 35 µl of an RNase solution [10 mg RNase (34390, Serva) in 1 ml milli-Q water]. Finally, 8µl DNA solution was mixed with 2 µl loading dye (4 g sucrose and 2.5 mg bromophenolblue dissolved in 6 ml TE buffer) and run on a 1% (w/v) agarose gel in 1x TAE buffer for 30 min at 100V to verify the DNA extraction. Quality of the DNA samples was verified by spectrophotometric measurements at 260/280/234 nm.

PCR. PCR was performed using a *Taq* polymerase kit (Applied Bio Systems, New Jersey, USA). Primers used in this study were those as described by Muyzer *et al*. (1993) amplifying the V3 region of bacterial 16S rDNA. The forward primer F357-GC contained the GC clamp (5'-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG-3') and had the following sequence : 5'-GC-clamp-TACGGGAGGCAGCAG-3'. The reverse primer 518R had the sequence: 5'-ATTACCGCGGCTGCTGG-3'. PCR reaction volumes of 50 µl contained 6µl of 10x PCR buffer containing 15 mM $MgCl_2$, 2.5 µl BSA , 2.5 µl dNTP's (2 mM each), 2 µl of each primer (5 µM), 0.25 µl Taq polymerase (5 units/µl), 33.75 µl sterile milliQ and 1 µl of 10-fold diluted DNA solution. The following PCR program was used : initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 20s, annealing at 55°C for 45s and extension at 72°C for 1 min; final extension at 72°C for 7 min followed by cooling to 4°C. PCR was

verified by mixing 8 µl of PCR product with 2 µl of loading dye and running it on a 2% (w/v) agarose gel for 30 min at 100V, flanked by the EZ Load 100bp Molecular Ruler (170-8352, Biorad) (data not shown).

DGGE analysis. PCR products were analysed on DGGE gels based on the protocol by Muyzer and co-workers (1993) applying the following modifications. Polyacrylamide gels (160 x 160 x 1 mm) consisted of 8% (v/v) polyacrylamide (EC-890, National Diagnostics, Atlanta, USA) in 1x TAE buffer (161-0773, Biorad, Hercules, USA). By diluting a 100% denaturing polyacrylamide solution [containing 7M urea (EC-605, National Diagnostics) and 40% formamide (F-9037, Sigma, St. Louis, USA)], with a 0% denaturing polyacrylamide solution (containing no denaturing components), the polyacrylamide solutions of desired denaturing percentage were obtained. In this study two types of denaturing gradients were used, namely a 35 – 70% gradient and a 40 – 55% gradient. The 24-ml gradient gels were casted using a gradient former (165-4120, Biorad) and a pump (731-8142, Biorad) set at a constant speed of 5 ml/min. Denaturing gels were allowed to polymerise for 3h after which a 5-ml non-denaturing stacking gel was poured on top containing a 16-well comb. After 1 h of polymerisation, PCR samples were loaded into the wells, and electrophoresis was performed for 16 h at 70 V in a 1x TAE buffer at a constant temperature of 60°C using the Dcode system (170-9081, Biorad). Gels were stained with ethidium bromide (solution of 50 µl EtBr in 500 ml TAE buffer) for 1 h, followed by visualisation of DGGE band profiles under UV light. Digital capturing was performed using the Foto/Analyst™ CCD Camera (Fotodyne Inc., Hartland, WI, USA) combined with the Iris Video Digitize™ software package (Inside Technology, Amersfoort, The Netherlands).

Processing of DGGE gels. For DGGE to be used as a direct identification method, a reference pattern was designed consisting of 6 different type strain V3 amplicons (**Fig. 1**). By running this reference pattern every 6 lanes on each DGGE gel, it became possible to digitally normalise the gel patterns by comparison with a standard pattern using the BioNumerics (BN) software package version 2.50 (Applied-Maths, St.-Martens-Latem, Belgium, http://www.applied-maths.com). This normalisation enables comparison of DGGE gels, provided that they consist of the same denaturing gradient. For all known probiotic species, the band position of their corresponding type strains was determined and stored in a BN database. The amplicons obtained from probiotic products were run on a DGGE gel and after normalisation based on the standard reference pattern of the BN database, individual bands in the product band pattern could be identified. Amplicons of isolates, identified with SDS-PAGE, corresponding to the species claimed on the product label, were run next to the amplicon of the probiotic product itself, as an additional visual confirmatory identification (**Fig 1**).

Results

Culture-dependent analysis of probiotic products. Results of the isolation and identification of probiotic strains from the tested products are presented in **Table 1**. Isolation was performed using four selective isolation media, followed by an identification using SDS-PAGE separation of whole-cell protein extracts and comparison of the species specific patterns with a laboratory-based identification library as described by Temmerman and co-workers (2003). Colony counts on the media used, were substantially lower in case of the freeze-dried products, yielding between 10^5 and 10^7 CFU/g of product, compared to the dairy products producing between 10^7 and 10^9 CFU/ml. Furthermore, from 6 products (i.e. Actimel, Vitamel, Aciforce, Bacilac, Bififlor and Proflora) not all species claimed on the labels could be isolated and two products (i.e. Bacilac and Vitamel) contained a probiotic strain belonging to another species than the ones mentioned on the label (**Table 1**).

Culture-independent analysis of probiotic products. For the purpose of cultivation-independent analysis, total bacterial DNA needed to be extracted directly from the product. This was done on a reproducible basis by adding a lysozyme and a number of centrifugation steps to the original protocol by Pitcher and co-workers (1989). The PCR program described in this paper was able to reproducibly amplify the V3 region of the 16S rDNA of all samples tested. Sometimes it was found that adding 2 µl of DNA to the PCR mixture instead of 1 µl was needed to enhance the intensity of some bands on the DGGE gels, in order to aid the visual interpretation of the results. For each of the 10 probiotic products, a 35-70% DGGE gel was run on which the following PCR amplicons were loaded next to each other : Probiotic product, artificial mixture of type strains simulating the species composition of the product, individual type strains of the species claimed on the label (**Fig. 1**). Every 6 lanes, all gels contained the reference pattern. A gel on which the amplicons of all 10 product DNAs were included is presented in **Fig 2.**

Table 1. Overview of culture-dependent and culture-independent analysis of 10 probiotic products

Product Name	Producer (Country)	Organisms stated on the product label	Organisms as detected by	
			Culture-dependent analysis (SDS-PAGE of proteins)	Culture-independent analysis (PCR-DGGE)
A. Dairy products				
Actimel	Danone (France)	*L. casei*, living yoghurt cultures	*L. casei*	*L. casei, L. bulgaricus, S. thermophilus*
Activia	Danone (France)	*Bifidobacterium*, living yoghurt cultures	*B. lactis, L. bulgaricus S. thermophilus, Lc. lactis*	*B. lactis, L. bulgaricus, S. thermophilus, Lc. lactis*
Vitamel	Campina (The Netherlands)	*L. casei* GG, *B. bifidum, L. acidophilus*	*L. acidophilus, L. rhamnosus*	*L. acidophilus, L. rhamnosus, S. thermophilus*
Yakult	Yakult (The Netherlands)	*L. casei*	*L. casei*	*L. casei*
B. Fruit Drink				
Provie	Skåne Mejerier (Sweden)	*L. plantarum*	*L. plantarum*	*L. plantarum*
C. Freeze-dried products				
Aciforce	Biohorma (The Netherlands)	*L. acidophilus, Lc. lactis, E. faecium B. bifidum*	*Lc. lactis, E. faecium*	*Lc. lactis, E. faecium, L. acidophilus, B. lactis*
Bacilac	THT (Belgium)	*L. acidophilus, L. rhamnosus*	*L. helveticus*	*L. helveticus, L. rhamnosus*
Bactisubtil	Synthelabo (Belgium)	*Bacillus* IP5832	*Bacillus cereus*	*Bacillus cereus*
Bififlor	Eko-Bio (The Netherlands)	*L. acidophilus, L. rhamnosus, B. bifidum*	Yeast	*L. acidophilus, L. rhamnosus*
Proflora	Chefaro (Belgium)	*L. acidophilus, L. bulgaricus, S. thermophilus, Bifidobacterium*	*L. acidophilus, S. thermophilus, B. lactis*	*L. acidophilus, S. thermophilus, B. lactis*

L. = Lactobacillus, B. = Bifidobacterium, S. = Streptococcus, E. = Enterococcus and *Lc. = Lactococcus.*

L. bulgaricus is shortened writing for *Lactobacillus delbrueckii* subsp. *bulgaricus*

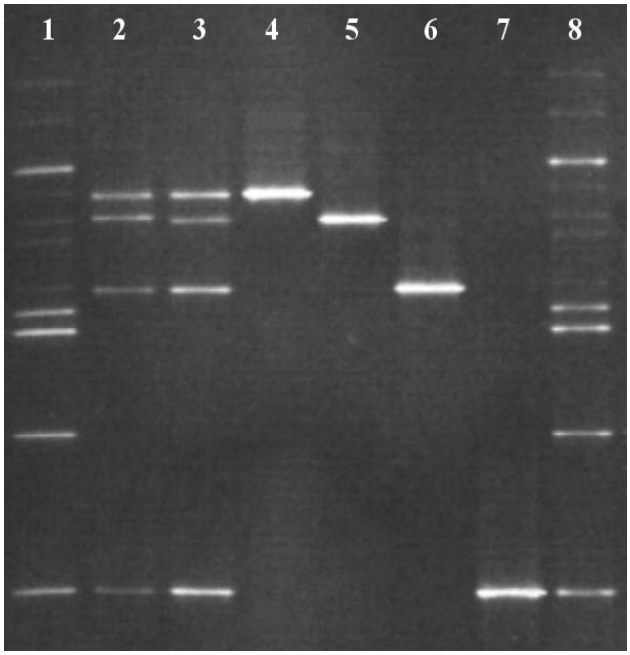

Fig 1. Example of a 35-70% DGGE gel for product analysis. Lane 1 and 8: reference pattern (composition of V3 amplicons of *Enterococcus solitarius, Enterococcus flavescens, Bacillus cereus, Lactobacillus rhamnosus and Bifidobacterium lactis*); lane 2: Aciforce amplicon; lane 3: amplicon from the cell suspension simulating Aciforce; lane 4: *Enterococcus faecium*; lane 5: *Lactobacillus acidophilus*; lane 6: *Lactococcus lactis*; lane 7: *Bifidobacterium lactis*.

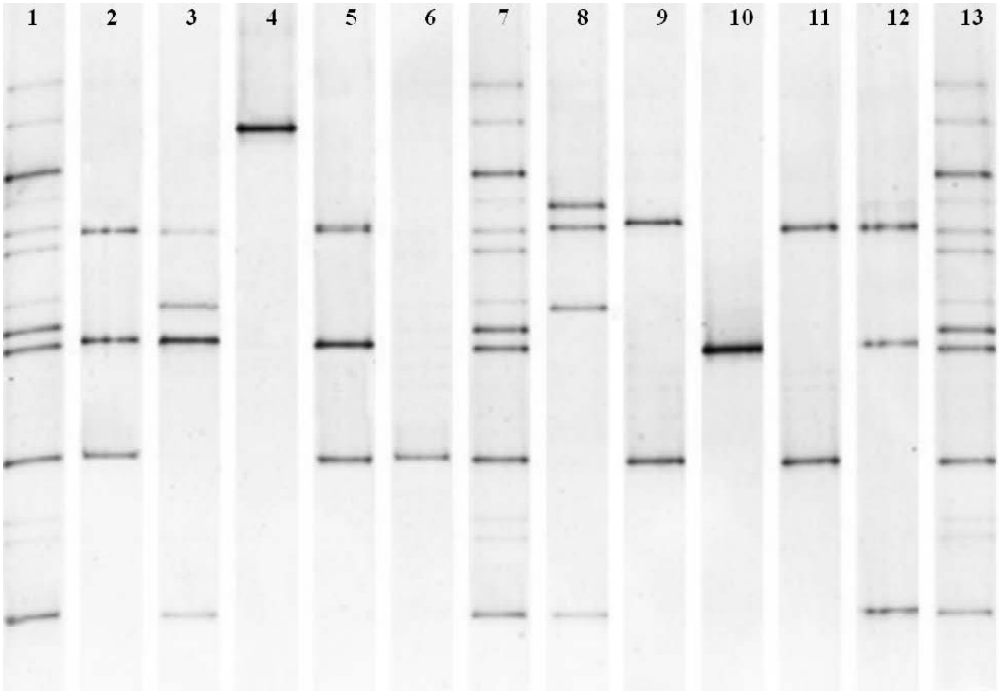

Fig 2. Normalised 35-70% DGGE gel showing the V3 amplicons of 10 probiotic products. Lane 1, 7 and 13: reference pattern, lane 2: Actimel; lane 3: Activia; lane 4: Provie; lane 5: Vitamel; lane 6: Yakult; lane 8: Aciforce; lane 9: Bacilac; lane 10: Bactisubtil; lane 11: Bififlor; lane 12: Proflora.

Identification was performed after normalisation of the gel using the standard reference pattern, followed by comparison of the band positions with those of identified type strains present in a newly built BN database. Verification of this identification was performed by running the V3 amplicon of type strains or isolates, originating from the culture-dependent analysis, on a DGGE gel next to the probiotic product amplicon. In a few cases, two phylogenetic closely related species produced an amplicon that could not be clearly separated on a 35-70% gradient gel. Therefore, DGGE gels with a more narrow 40-55% denaturing gradient were run, to obtain a higher band position resolution. As shown in **Fig. 3**, the amplicons representing *Lactobacillus delbrueckii* subsp. *bulgaricus* and *Lactobacillus acidophilus* may be confused with each other on the 35-70% gradient gel (**Fig. 3a**), but can be clearly separated electrophoretically using a 40-55% gradient (**Fig. 3b**).

Results of the culture-independent DGGE analysis of the 10 probiotic products, compared with the results of the culture-dependent analysis, are presented in **Table 1**. Two different scenarios were found. DGGE analysis of 5 products (i.e. Activia, Yakult, Provie, Bactisubtil and Proflora) detected the same species as with conventional isolation procedures. In the remaining 5 products (i.e. Actimel, Vitamel, Aciforce, Bacilac and Bififlor), DGGE analysis was able to detect more claimed species than recovered by isolation. From two products (i.e. Bacilac and Vitamel), another species than those mentioned on the label could be isolated and identified, which was confirmed by DGGE analysis.

The detection limit of the DGGE method was also determined by preparing a 10-fold serial dilution of a pure culture of *Lactobacillus rhamnosus* (LMG 18243) in PPS. After plating 100 µl of each dilution on MRSA medium for incubation during 48h at 37°C aerobically, DNA was extracted from each dilution and PCR-DGGE analysis was performed. It was found that this technique produced a clear band corresponding down to 10^4 CFU/ml. To determine the reproducibility of the technique, 3 different batches of each product were analysed at different points of time. In all cases, identical results were obtained (data not shown).

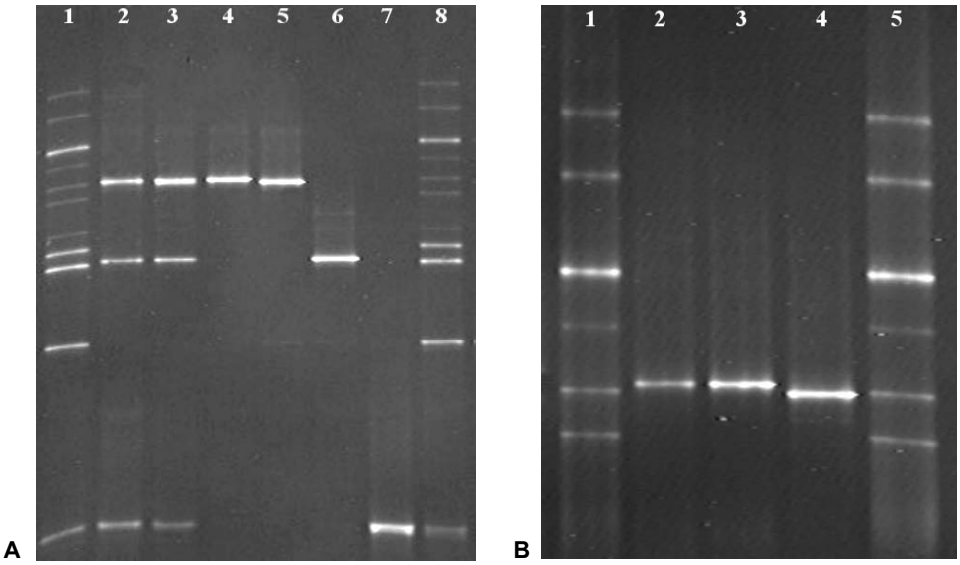

Fig 3a. A 35-70% DGGE gel showing analysis of Proflora. Lane 1 and 8: Reference pattern; lane 2: Proflora; lane 3: Cell suspension simulating Proflora; lane 4: *Lactobacillus acidophilus*; lane 5: *Lactobacillus delbrueckii* subsp. *bulgaricus*; lane 6: *Streptococcus thermophilus*; lane 7: *Bifidobacterium lactis*. The difference in band position of *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus* is not well pronounced on this 35-70% gradient gel.

Fig 3b. A 40-55% DGGE gel focussing in on the difference in band position between *L. acidophilus* and *L. delbrueckii* subsp. *bulgaricus*. Lane 1 and 5: Reference pattern; lane 2: Proflora; lane 3: *L. acidophilus*; lane 4: *L. delbrueckii* subsp. *bulgaricus.*

Discussion

Despite the expansion of the probiotic market (Stanton *et al*., 2001) and the accompanying scientific research (Naidu *et al*., 1999), a number of recent reports clearly highlighted the poor quality of many probiotic products concerning their content and label information (Hughes *et al*., 1990, Hamilton-Miller *et al*., 1996, 1999; Yeung *et al*., 2002, Temmerman *et al*., 2003). In relation to their safety and functionality, it is of major importance that these products are correctly labelled and contain well-documented probiotic strains (Sanders and Huis in't Veld, 1999). In this study, the culture-independent DGGE method was compared with a culture-dependent procedure for the detection and identification of the constituting strains in probiotic products. As also demonstrated in a previous paper (Temmerman *et al*., 2003), it was found that the numbers of bacteria isolated from the freeze-dried products were substantially lower as compared to the dairy products and the fruit drink. Furthermore, 6 products were not found to contain all the claimed species as determined with the culture-dependent analysis. Next to the fact that some of these products may have been mislabelled or display a low production quality, the poor recovery results might to some extent also be ascribed to the inherent selectivity of the isolation media used. Previous studies have already stressed the need for culture-independent methods to circumvent the limitations of conventional cultivation (Ampe *et al*., 1999; Ercolini *et al*., 2001).

As part of such a culture-independent method, a reliable DNA extraction and PCR method needs to be performed. By preparing artificial mixtures of type strains to simulate the species composition of the analysed products, we were able to confirm the suitability of our DNA extraction and PCR method applied to the products. So far, identification of DGGE bands has not been performed without additional steps such as gel extraction and sequencing (Ercolini *et al*., 2001). By means of a reference pattern included in each gel, combined with the BioNumerics software, it was possible to create a database containing all band positions of type strains representing probiotic species. Following digital normalisation of the gel by comparison of the reference patterns with the standard pattern of the database, it was possible to assign an identity to each band in the band pattern representing the probiotic products. This identification as determined with DGGE could be confirmed by means of co-running amplicons of pure cultures, previously identified using protein profiling. Furthermore, multiple probiotic isolates from a certain species produced bands, which coincide with the band position

of the type strain amplicon, indicating that banding patterns are species- specific. However, in case of some phylogenetic closely related species (Schleifer and Ludwig, 1995) differences in band position between two species may sometimes be too small on a 35-70% gel in order to obtain a clear-cut identification. This could be solved by applying a more narrow denaturing gradient, enlarging the difference in band position. Alternatively, the use of other primers might result in amplicons, which are readily separated, from each other on DGGE, thereby making it possible to identify species producing overlapping bands with the V3 primers. However, every change concerning the gradient, primer set or electrophoresis conditions results in the necessity to build a new database corresponding to these new parameters. This implies that the use of DGGE as a direct identification method will only be successful with rather simple microbial ecosystems such as probiotic products. With the rising complexity of a microbial ecosystem, it becomes necessary to change more and more parameters, making the method increasingly time-consuming. As such, Ercolini and co-workers (2001) studied the potential of DGGE to analyse natural whey cultures for cheese production, but found it necessary to sequence the bands in the DGGE profile. In contrast, probiotic products can be regarded as ecosystems developed from well-controlled fermentations with a low taxonomic diversity. In the current study, analysis of type strains representing known probiotic species never resulted in two species with identical band position. Moreover, from all species investigated, only *Lactobacillus reuteri* produced multiple bands on a DGGE gels (data not shown). This species was not included in any of the tested products, but a previous study (Temmerman *et al*., 2003) showed that out of 55 probiotic products, only 2 products contained this species.

When comparing the results of the culture-dependent and culture-independent analysis of probiotic products, it can be concluded that DGGE has a much higher detection and identification potential. Whereas conventional isolation revealed that 6 out of 10 products did not contain the species claimed on their labels, DGGE analysis was able to detect additional species in 5 of these 6 products. Nevertheless, 4 products (i.e. Vitamel, Bacilac, Bififlor and Proflora) were considered to have incorrect labels after analysis with both approaches. This indicates that the previously reported poor product quality of probiotics (Hughes *et al*., 1990, Hamilton-Miller *et al*., 1996, 1999; Yeung *et al*., 2002, Temmerman *et al*., 2003) can not solely be attributed to shortcomings of cultivation-based methods. Evaluation of 3 different batches of all products indicated that DGGE analysis is very reproducible, since in all cases the same bacterial species were detected. This was not the case for the culture-dependent approach, where one product produced a species previously not detected from another batch of the product. Mainly detection of bifidobacteria impairs the reproducibility of the culture-dependent

approach, because of the lack of suitable selective isolation media (Roy, 2001). A potential drawback of the DGGE approach may be that no information is obtained concerning the level of bacterial viability in probiotic products, implying that culture-dependent analysis may still add valuable information. Also, the detection limit of 10^4 CFU/ml as determined by this study may result in the failure to detect species that are present in lower numbers. In this regard, it can be seriously questioned whether organisms present in such low numbers can exert any significant probiotic effect at all. In the near future, the linkage of real-time PCR to the DGGE method may result in a very powerful tool for both the qualitative and quantitative analysis of all kinds of (bacterial) fermentation products. As was reported previously by Temmerman and co-workers (2003), this paper again demonstrates that a substantial percentage of probiotic products suffers from incorrect labels and low counts. Numerous studies (Naidu *et al*., 1999) demonstrated different probiotic effects exerted by different bacteria, but how can the consumer select the product containing the most suitable strain for his/her symptons if the product labels are incorrect, or the strains or absent? Nevertheless, this study clearly demonstrates DGGE to be a fast, reliable and reproducible culture-independent approach for analysis of probiotic products, with higher detection and identification potential than conventional culture-dependent analysis.

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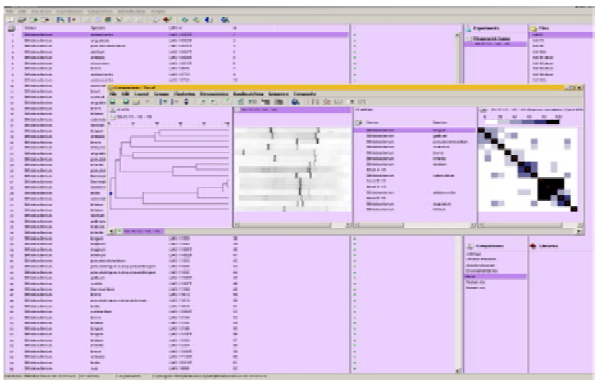
5.2. Nested-PCR DGGE for the microbial analysis of bifidobacterial communities



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Summary

The taxonomic characterization of bacterial communities is difficult to combine with the monitoring of its temporal changes. None of the currently available identification techniques are able to visualise a 'complete' community, whereas techniques designed for analysing bacterial ecosystems generally display a limited or labour-intensive identification potential. This paper describes the optimisation and validation of a nested PCR-DGGE approach for the species-specific analysis of bifidobacterial communities from any ecosystem. The method comprises a *Bifidobacterium*-specific PCR step, followed by purification of the amplicons that serve as template DNA in the second PCR step amplifying the V3 and V6-V8 region of the 16S rRNA gene. A mix of both amplicons is analysed on a Denaturing Gradient Gel Electrophoresis (DGGE) gel, after which the band positions are compared with a previously constructed database of reference strains. The method was validated through the analysis of four artificial mixtures mimicking the possible bifidobacterial microbiota of the human and chicken intestine, rumen and environmental sample, and of two fecal samples. Except for the species *B. coryneforme* and *B. indicum*, all currently known bifidobacteria originating from various ecosystems can be identified in a highly reproducible manner. Because no further cloning and sequencing of the DGGE bands is necessary, this nested PCR-DGGE technique can be completed within a 24 hours span, allowing the species-specific monitoring of temporal changes in the bifidobacterial community.

Introduction

The genus *Bifidobacterium* consists of Gram-positive bacteria with a %G+C content above 50, currently enclosing over 30 species (Hoyles *et al*., 2002). The main habitat of bifidobacteria is the human and animal intestinal tract (Crociani *et al*., 1996; Matsuki *et al*., 1999; Sghir *et al*., 2000) although sewage (Scardovi *et al*., 1979), anaerobic digesters (Dong *et al*., 2000) and fermented milk (Meile *et al*., 1997) have also been reported as isolation sources of certain *Bifidobacterium* species. The past decade has witnessed a fast growing interest in bifidobacteria, mainly because of the health-promoting properties of certain species (Ballongue *et al*., 1995; Goldin *et al*., 1992). Because of their growing application in probiotic dairy products and dried food supplements (Stanton *et al*., 2001), many of the recent studies emphasize only on the intestinal bifidobacteria (Matsuki *et al*., 1999; Satokari *et al*., 2001; Requena *et al*., 2002).

Until recently, routine identification of bifidobacteria was mainly based on phenotypic characterization, often leading to conflicting or doubtful results. Molecular techniques such as ARDRA (Hall *et al*., 2001; Ventura *et al*., 2001), 16S rRNA gene sequencing (Hoyles *et al*., 2002), FISH (Ventura *et al*., 2001), SDS-PAGE of cellular proteins, RAPD-PCR, PFGE, dot blot hybridisations (Reuter *et al*., 2002) and rep-PCR (Masco *et al*., 2003), have been evaluated and optimized for identification of bifidobacterial pure cultures to the species or even to the strain level. However, numerous situations call for the direct species-specific detection of bifidobacteria in microbial ecosystems in relation to temporal and environmental changes. Because of their culture-dependent nature, most of the above mentioned techniques are not suitable for this purpose. Therefore, culture-independent methods have been designed, of which Denaturing Gradient Gel Electrophoresis (DGGE) is the most commonly used technique. The DGGE principle relies on the electrophoretic separation of PCR amplicons of equal length in a sequence-specific manner (Muyzer *et al*., 1993, 1998). When universal bacterial PCR primers are used, only the dominant microbiota of an ecosystem will be visualised on a DGGE gel (Zoetendal *et al*., 1998), producing complex banding patterns. In case identification of these bands is desired, additional cloning and sequencing of the extracted bands is required (Ercolini *et al*., 2001; Satokari *et al*., 2001; Favier *et al*., 2002). However, these extra steps render the method laborious and time-consuming, impairing the potential of DGGE as a fast method for

bacterial population fingerprinting. In this regard, the use of species- or genus-specific primers represents a major step forward, usually resulting in less complex DGGE banding patterns that only display the diversity of a specific bifidobacterial group within the targetted ecosystem (Kok *et al*., 1996; Kaufmann *et al*., 1997; Matsuki *et al*., 1999; Requena *et al*., 2002). Most of these studies reported the detection of a limited number of mainly intestinal bifidobacterial species, although cloning and sequencing steps were still needed in order to confirm the detection results.

In an attempt to enhance the operational time of culture-independent detection of bifidobacteria, this paper describes the optimisation and validation of a nested PCR-DGGE approach for the direct identification of all currently known bifidobacterial species present in ecosystems with a variable degree of complexity, including both artificial and natural samples.

Material and Methods

Strain collection. All strains used in this study were obtained from the BCCM™/LMG bacteria collection (http://www.belspo.be/bccm/lmg.htm) (**Fig 2**). All strains were grown for 24h at 37°C under anaerobic conditions (80% N_2, 10% H_2, 10% CO_2) on Modified Columbia Agar (MCA) comprising 23g special peptone (L72, Oxoid, Basingstoke, UK), 1g soluble starch (1.01252.0250, Merck, Darmstadt, Germany), 5g NaCl, 0.3g cystein-HCl-H_2O (C4820, Sigma, Bornem, Belgium), 5g glucose (500520-887, Vel, Leuven, Belgium) and 15g agar (L11, Oxoid) dissolved in 1 liter of distiled water.

Total DNA preparation. Extraction of total bacterial DNA from pure cultures was based on the method described by Pitcher and co-workers (1989) with modifications regarding the concentration of lysozyme and an additional RNase step at the end. Total DNA was extracted from cells harvested from a 24h culture, grown on MCA, at 37°C under anaerobic conditions. The cells (half a loop) were washed in 500 µl TE-buffer (1mM EDTA pH 8.0; 10mM Tris-HCl pH 8.0), after which the cells were collected by centrifugation during 2 min at 13000 rpm. Following the removal of the supernatant, the resulting pellet was then frozen at –20°C for 1 h to facilitate the rupture of the Gram-positive cell wall. The thawed pellet was suspended in 150µl lysozyme-solution [5 mg lysozyme (SERVA, # 28262, Heidelberg, Germany) in 150 µl of TE buffer], followed by incubation at 37°C during 40 min. The remaining steps of the procedure were performed according to the protocol of Pitcher and co-workers (17). The resulting DNA pellet was then dissolved in 200 µl TE-buffer overnight at 4°C after which an RNA digesting step was performed by adding 2 µl of RNase solution [10 mg RNase (SIGMA, # R6513) dissolved in 1 ml milli-Q water] followed by a 90 min incubation step at 37°C. Finally, 8 µl of DNA solution was mixed with 2 µl loading dye and run on a 1% (w/v) agarose gel in 1x TAE buffer for 30 min at 100V to verify the DNA extraction. Quality of the DNA samples was verified by spectrophotometric measurements at 260/280/234 nm.

DNA extraction from fecal samples was also based on the protocol by Pitcher and co-workers 1989), with other modifications. Upon collection of the fecal samples, 700 mg (wet weight) was homogenized in 9,3 ml physiological phosphate buffer. One ml of the fecal sample suspension was transferred to an Eppendorf tube and centrifuged for 5 min at 13000 rpm. After removal of the supernatans, the pellet is resuspended in 1 ml TE buffer and is again centrifuged for 5 min at 13000 rpm. After removal of the supernatans, the pellet is resuspended in 150 µL enzyme solution to degenerate the bacterial cell wall. Per sample, this enzyme mix consists of 6 mg lysozyme powder and 40 µL mutanolysine dissolved in 110 µL TE buffer. Further steps are according to the protocol of Pitcher and co-workers (1989).

Nested PCR. A schematic overview of the method is presented in **Fig. 1**. All PCR reactions were performed using a *Taq* polymerase kit (Applied BioSystems, New Jersey, USA). The first PCR applied primers lm26-f and lm3-r described by Kaufmann and co-workers (1997), amplifying a 1417 bp fragment of bifidobacterial 16S rDNA (**Table 1**). PCR reaction volumes of 50 µl contained 8 µl of 10x PCR buffer (incl. 15 mM $MgCl_2$), 3.5 µl BSA , 3.5 µl dNTP's (2 mM each), 3 µl of each primer (5 µM), 0.35 µl *Taq* polymerase (5 units/µl), 27.65 µl sterile milliQ and 1 µl of 10-fold diluted DNA solution. The following PCR program was used : initial denaturation at 94°C for 5 min; 3 cycles of denaturation at 94°C for 45s, annealing at 55°C for 2 min and extension at 72°C for 1 min; 30 cycles of denaturation at 94°C for 20s, annealing at 55°C for 1 min and extension at 72°C for 1 min; final extension at 72°C for 7 min followed by cooling to 4°C. PCR was verified by mixing 8 µl of amplicon with 2 µl of loading dye and running it on a 1% (w/v) agarose gel for 30 min at 100V, flanked by the EZ Load 100bp Molecular Ruler (170-8352, Biorad). In order to eliminate remaining oligonucleotides and original template DNA, purification of the amplicons was performed using the QIAquick PCR Purification Kit (28104, Qiagen, Hilden, Germany) according to the manufacturer's instructions. Subsequently, a second PCR was performed using the amplicons of the first PCR as template DNA. Because of the length of the first amplicon (positions 15 to 1432), different primer pairs can be used for the second PCR, depending on the desired application.

This study made use of two sets of primers (**Table 1**). The first primer set (F357-GC/518R) amplifies the V3 region of bacterial 16S rDNA (Muyzer *et al*., 1993), whereas the second set of primers (U968F-GC/L1401R) targets the V6 to V8 region of bacterial 16S rDNA (Zoetendal *et al*., 1998). In both cases, the forward primer contained a GC clamp to facilitate separation of the amplicons on a DGGE gel. For both primer sets, the PCR reaction volumes of 50 µl contained 6µl of 10x PCR buffer containing 15 mM $MgCl_2$, 2.5 µl BSA , 2.5 µl dNTP's (2 mM each), 2 µl of each primer (5 µM), 0.25 µl *Taq* polymerase (5 units/µl), 33.75 µl sterile milliQ and 1 µl of 10-fold diluted DNA solution. The following PCR program was used : initial denaturation at 94°C for 5 min; 30 cycles of denaturation at 94°C for 20s, annealing at 55°C for 45s and extension at 72°C for 1 min; final extension at 72°C for 7 min followed by cooling to 4°C. PCR was verified by mixing 8 µl of amplicon with 2 µl of loading dye and running it on a 2% (w/v) agarose gel for 30 min at 100V, flanked by the EZ Load 100bp Molecular Ruler (170-8352, Biorad).

Fig. 1: Overview of the nested PCR-DGGE technique.

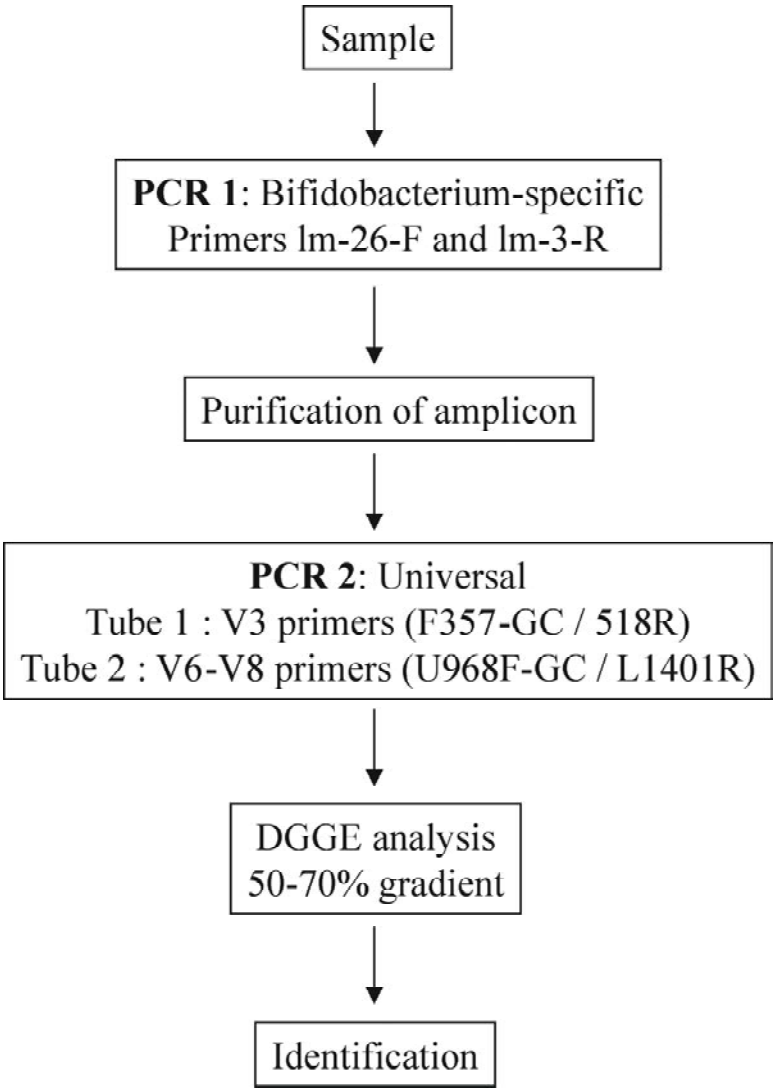

Table 1. Primers used in this study.

Primer	Target	Position	Sequence	Reference
lm-26f	*Bifidobacterium* 16S rDNA	(15-35)	5'-GATTCTGGCTCAGGATGAACG-3'	Kaufmann *et al*., 1997
lm-3r	*Bifidobacterium* 16S rDNA	(1412-1432)	5'-CGGGTGCTICCCCACTTTCATG-3'	Kaufmann *et al*., 1997
F357-GC	Bacterial 16S rDNA V3 region	(341-357)	5'-GC-clamp-GCCTACGGAGGCAGCAG-3'	Muyzer *et al*., 1993
518-R	Bacterial 16S rDNA V3 region	(518-534)	5'-ATTACCGCGGCTGCTGG-3'	Muyzer *et al*., 1993
U968F-GC	Bacterial 16S rDNA V6-V8 region	(968-985)	5'-GC-clamp-AACGCGAAGAACCTTAC-3'	Zoetendal *et al*., 1998
L1401R	Bacterial 16S rDNA V6-V8 region	(1401-1418)	5'-GCGTGTGTACAAGACCC-3'	Zoetendal *et al*., 1998

GC-clamp = 5-CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGG-3

DGGE. PCR products were analysed on DGGE gels based on the protocol by Muyzer and co-workers (1993, 1999) with modifications according to Temmerman and co-workers (2003). Because of the high %GC content of bifidobacteria, gels with a 50-70% denaturing gradient were used. Gels were stained with ethidium bromide for 15 min, followed by visualisation of DGGE band profiles under UV light. Digital capturing was performed using the Foto/Analyst^{TM} CCD Camera (Fotodyne Inc., Hartland, WI, USA) combined with the Iris Video Digitize^{TM} software package (Inside Technology, Amersfoort, The Netherlands). During the analysis and database construction using pure cultures, the V3 and V6-8 amplicons were mixed and loaded in the same lane prior to electrophoresis, whereas the analysis of bifidobacterial mixtures and ecosystems required the two amplicons to be loaded separately in adjacent lanes.

Gel processing. For direct identification of bands in a given DGGE profile, a database was created containing the V3 and V6-V8 band positions of bifidobacterial type and reference strains (**Fig. 2**), using the BioNumerics (BN) software package version 2.50 (Applied-Maths, St.-Martens-Latem, Belgium, http://www.applied-maths.com). By mixing several PCR amplicons, a reference pattern was designed consisting the V3 amplicons of 4 different type strains. By including this reference pattern every 5 lanes on each DGGE gel, it is possible to digitally normalise the in-between lying banding patterns by aligning each reference lane with the standard reference pattern defined in the BN database. This normalisation enables comparison of banding patterns originating from different DGGE gels, provided that they comprise the same reference pattern ran under identical electrophoretic conditions. Following normalization, the identity of bifidobacteria present in any environmental sample was determined by comparing the band positions in the sample profile with the BN database.

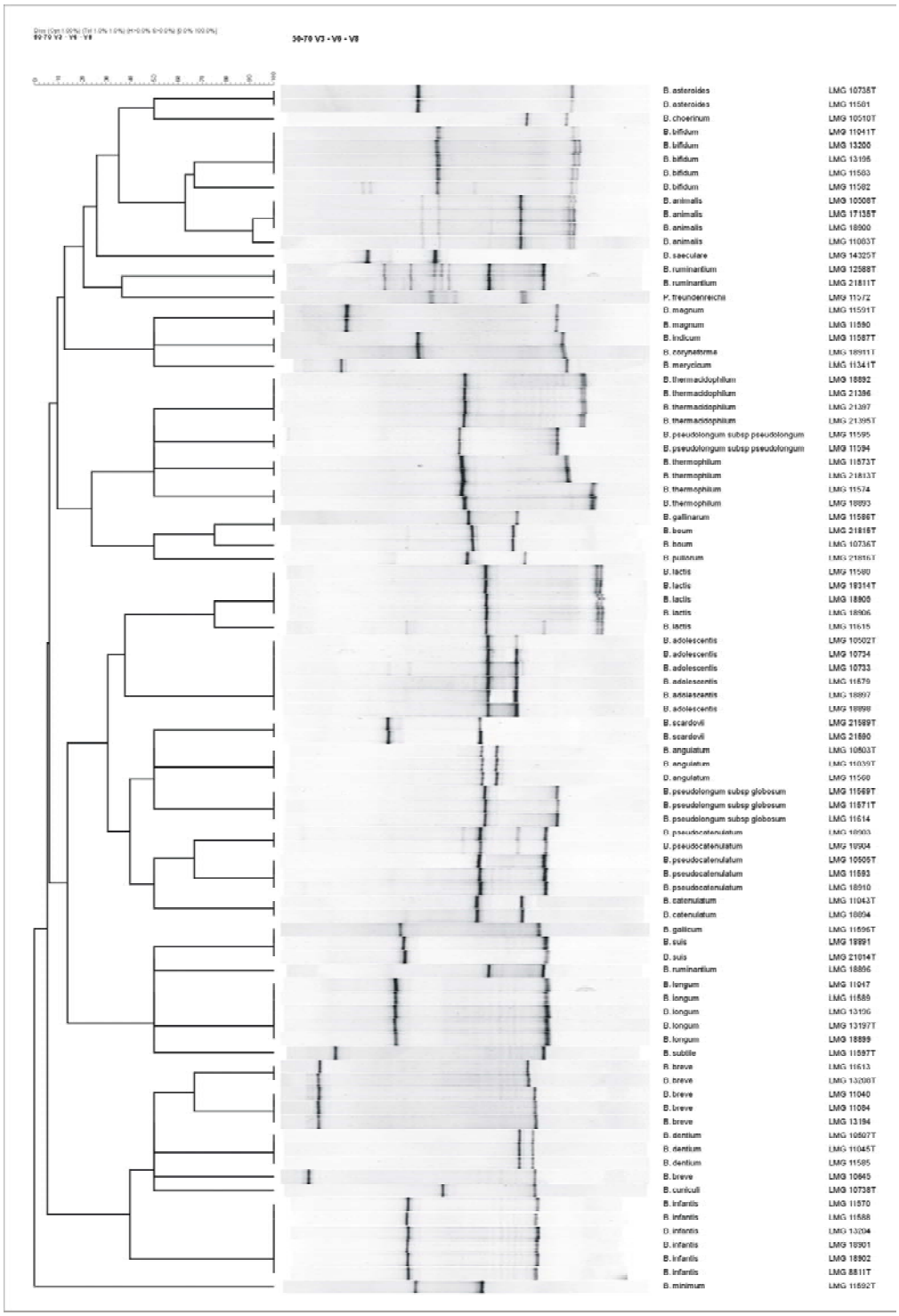

Fig. 2. Dendrogram showing the normalized band positions of bifidobacterial reference strains. Numbers behind the species assignment stand for the BCCM™/LMG accession number.

Results

Nested PCR. The nested PCR approach described in this study for the identification of bifidobacteria in various ecosystems applies a first PCR step using the genus-specific primers Im-3 and Im-26 (**Table 1**), resulting in an amplicon for all bifidobacterial reference strains (**Fig. 1**). Also a broad range of non-target organisms was tested and in contrary to the results of Kaufmann and co-workers (1997), weak PCR signals for *Propionibacterium freudenreichii* and *Gardnerella vaginalis* were obtained. The bifidobacterial amplicons could not be analysed directly on a DGGE gel because their length of 1417 bp by far exceeds the 500 bp limit for DGGE analysis. The advantage of generating amplicons of this length is that they can serve as template DNA for other 16S rRNA gene primers such as the V3 primer combination F357-GC/518-R (**Table 1**) during the second PCR step. Because of the universal nature of these primers, a purification of the amplicons from the first PCR was performed in order to remove all remaining non-bifidobacterial template DNA. Analysis of the V3 amplicons on a DGGE gel showed that not all bifidobacterial species could be separated from each other, necessitating the additional use of a second universal primer set. For this purpose, we opted for the U968F-GC and L1401R primers (**Table 1**) targetting the V6-V8 region of the 16S rRNA gene. Because the same temperature program could be used, amplification of the V3 and V6-V8 regions during the second PCR step could be performed in the same run, although each in a separate PCR tube (**Fig 1**). For all bifidobacterial species, an amplicon was obtained for both the V3 and V6-V8 region (data not shown).

DGGE analysis of V3 and V6-V8 amplicons. Because of the high %GC content of bifidobacteria, the conventional 35-70% denaturing gradient was replaced by a 50-70% denaturing gradient. For some species (e.g. *B. lactis* and *B. pseudolongum* subsp. *globosum*), the band position of the V3 amplicon was indistinguishable (the left-hand band in each lane, **Fig. 2**), whereas for other bifidobacteria (e.g. *B. longum* and *B. pseudocatenulatum*), identical band positions were found for the V6-V8 amplicon (the right-hand band in each lane, **Fig. 2**). However, clustering analysis of the combined DGGE profile of both amplicons (V3-V6-V8) allowed to differentiate all bifidobacteria according to their (sub)species designation, except for the species *B. indicum* and *B. coryneforme* (**Fig. 2**). Although most V3-V6-V8 DGGE profiles consisted of two strong bands, one or more additional weak bands were noticed for *B. adolescentis, B. pseudocatenulatum, B. animalis* and *B. ruminantium*. Furthermore, in case of *B. thermophilum* and *B. breve*, two or three different combinations of V3 and V6-V8 band positions exist, respectively, due to slightly different band positions among certain strains.

Due to its low %G+C, the amplicon of *G. vaginalis* did not enter the 50-70% DGGE gel, whereas the band positions of *P. freundenreichii* could clearly be separated from those of all bifidobacteria (**Fig. 2**).

Artificial mixtures and fecal samples. The discriminatory potential of the V3-V6-V8 DGGE technique was further validated using 4 artificial mixtures of bifidobacterial DNA (**Table 2**), mimicking a human intestine, chicken intestine, rumen and environmental ecosystem (sewage), respectively, and by means of two human fecal samples. In order to avoid overlap of the V3 and V6-V8 band positions, both types of amplicons were loaded separately in two adjacent lanes on the DGGE gel. After normalisation, band positions of both amplicon types are compared with the BN database of reference strain. Clustering analysis of mixed community profiles using the BioNumerics software enabled identification of all bifidobacterial species present in all mixtures. Species with highly similar or identical V3 band positions can be further differentiated by comparison with the band positions of the V6-V8 amplicons and vice versa. **Fig. 3** shows the resulting identification of bands in the 4 artificial mixtures mimicking the possible bifidobacterial microbiota of (**A**) the human intestine, (**B**) rumen, (**C**) chicken intestine and (**D**) an environmental sample. These four mixtures clearly demonstrate the need to analyse both the V3 as well as V6-V8 amplicons. Overall, a detection limit of 10E4 CFU/ml was established and no preferential amplification was noticed resulting from various bacterial concentrations in the mixtures tested.

Finally, the method was also validated for characterization of bifidobacterial species present in two fecal samples originating from two volunteers (**Fig. 3A**). Fecal sample A contained five bifidobacterial species (*B. adolescentis, B. bifidum, B. catenulatum, B. gallicum* and *B. infantis*), whereas fecal sample B contained four species (*B. adolescentis, B. angulatum, B. bifidum* and *B. catenulatum*). Although some bands are less intense compared to those of the artificial mixtures or pure cultures, all bands could clearly be linked to *Bifidobacterium* species. Furthermore, there were no bands present in the fecal lanes, which could not be assigned to any of the bifidobacterial species.

Table 2. Microbial composition of four artificial mixtures mimicking bifidobacterial ecosystems

Mix	Mimicks	Species composition
1	Human intestine	*B. angulatum, B. bifidum, B. adolescentis, B. infantis, B. longum, B. breve, B. catenulatum, B. dentium, B. gallicum, B. pseudocatenulatum, B. scardovii*
2	Rumen	*B. ruminantium, B. pseudolongum* subsp. *pseudolongum, B. merycicum, B. thermophilum, B. boum*
3	Chicken intestine	*B. pullorum, B. pseudolongum* subsp. *pseudolongum, B. animalis, B. thermophilum, B. gallinarium*
4	Sewage	*B. thermacidophilum, B. minimum, B. subtile*

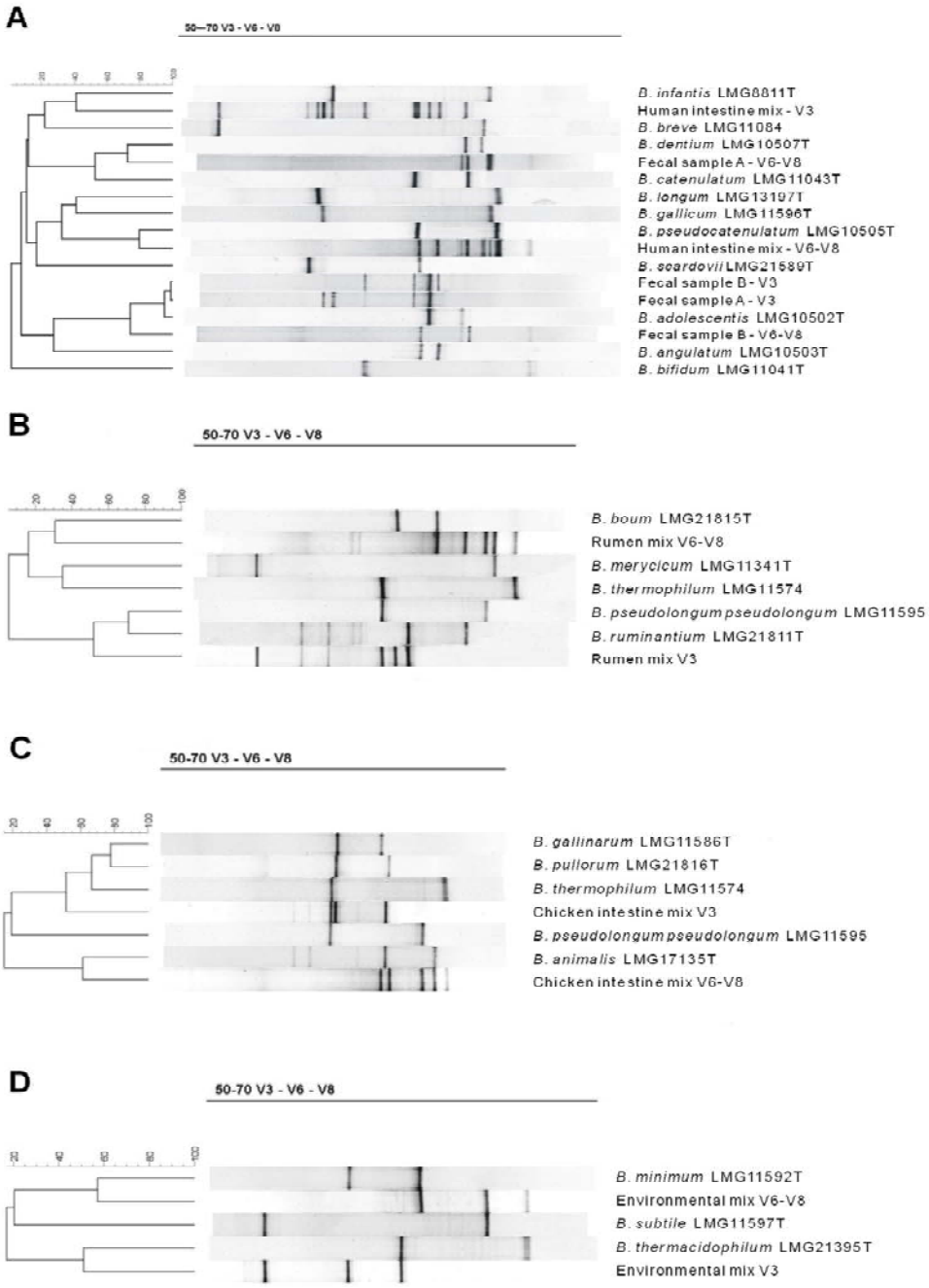

Fig 3. Use of the BioNumerics software for the identification of bifidobacteria present in 4 artificial mixtures, mimicking A: Human intestine, B: Rumen, C: Chicken intestine and D: Environmental sample. Fig. 3A also contains the V3 and V6-V8 profiles of two fecal samples. Fecal sample A contains *B. adolescentis, B. bifidum, B. catenulatum, B. gallicum* and *B. infantis* and fecal sample B contains *B. adolescentis, B. angulatum, B. bifidum* and *B. catenulatum.*

Discussion

At present, DGGE is the most applied technique to analyse bifidobacterial ecosystems, although cloning and sequencing of the DGGE-bands is still necessary to obtain a reliable identification (Ampe *et al*., 1999; Ercolini *et al*., 2001; Satokari *et al*., 2001; Favier *et al*., 2002). Based on previous research on DGGE analysis of probiotic products (Temmerman *et al*., 2003), the current study describes the design and validation of a nested PCR-DGGE method for the direct identification of currently known bifidobacteria present in natural or industrial ecosystems.

Until now, *Bifidobacterium*-specific primers suitable for DGGE, which allow the direct identification of all bifidobacteria, have not been described in literature. Therefore, a nested PCR approach was applied, combining a first genus-specific PCR step with a second universal PCR step. In between, a purification of the amplicons was necessary to remove small remaining fractions of non-bifidobacterial DNA. Amplification of the 16S rRNA gene V3 region alone did not allow the complete differentiation of all bifidobacteria, necessitating the combination of both the V3 and V6-V8 regions of the 16S rRNA gene. Because both primer sets can be used during the same PCR run, though in separate tubes, only a limited amount of extra work was required. For all bifidobacteria tested, both genus-specific and universal primers produced sufficient amounts of amplicon. Separation of the V3 and V6-V8 amplicons on a 50-70 % DGGE gel resulted in a clear identification of all bifidobacteria, except for *B. coryneforme* and *B. indicum*, which displayed identical band positions for both amplicons. The fact that these two species, originating from the intestinal tract of two different species of bees (Scardovi and Trovatelli, 1969), cannot be differentiated is in line with the rep-PCR data of Masco and co-workers (2003). After analysis of 6 different *B. breve* reference strains, it was found that 3 different combinations of V3 and V6-V8 band positions occurred. Likewise, two different combinations were observed among 5 *B. thermophilum* reference strains (**Fig. 2**). Probably, this is due to minor sequence variations within these species. As none of the combinations coincided with other species, this did not impair the identification potential of the method. This, together with the fact that both subspecies of *B. pseudolongum* could be readily distinguished, indicates that DGGE has an identification potential up to the subspecies level. Another observation was that *B. adolescentis, B. animalis, B. pseudocatenulatum* and *B. ruminantium* displayed additional weak bands for both 16S rRNA gene regions, possibly as a result of operon

heterogeneity, as previously observed for different genera (Muyzer *et al*., 1993; Zoetendal *et al*., 1998). This heterogeneity does not impair the identification potential of DGGE, because these additional bands are consistent among different strains of a specific taxon and readily recognizable. As already observed during a previous study (Temmerman *et al*., 2003), the V3 and V6-V8 amplicons of *B. animalis* and *B. lactis* have completely different band positions with *B. animalis* also showing operon heterogeneity, which indicates that both taxa are probably not belonging to the same species, as confirmed by Masco and co-workers (2003).

Because this technique has been designed and optimized for the analysis of (complex) mixtures of bifidobacteria, it was validated by means of four representative artificial mixtures of bifidobacteria and two fecal samples. For these mixtures, a perfect identification of all bifidobacteria present was possible. Because some band positions of the V3 region coincide with the V6-V8 band position of other bifidobacteria, it is necessary to load the two different amplicons in two adjacent lanes, also preventing banding patterns of becoming too complex. Because most of the bifidobacterial ecosystems also contain non-bifidobacteria, great care should be taken in the evaluation of the specificity of the approach. In addition to the selectivity of the genus-specific PCR and the purification of the amplicons, also the use of a 50-70% DGGE gel prevents amplicons from many non-bifidobacteria with a denaturation point between 35 and 50% denaturant, from entering the gel. This was demonstrated by the fact that *Gardnerella vaginalis* did not produce any bands on the 50-70% denaturing gel because of its %G+C content lower then 50. The only non-bifidobacterial species besides *G. vaginalis* known so far to produce an amplicon using the *Bifidobacterium*-specific primers, namely *Propionibacterium freundenreichii,* displays clearly separated band positions on the DGGE gels, thereby not impairing the identification potential of the technique. The bifidobacterial specificity of the technique was further demonstrated through the analysis of two fecal samples. Besides the 4-5 bifidobacterial species detected, respectively, no other bands were present on the gel that could not be linked to a certain bifidobacterial species. The fact that some bands were less intense compared to the artificial mixtures is due to the fact that in natural ecosystems different bifidobacterial species are present in various concentrations. However, it cannot be guaranteed that the optimized nested-PCR DGGE technique was capable of detecting all bifidobacteria present in the fecal samples. In this regard, optimization of certain procedure steps such as DNA extraction might be necessary depending on the ecosystem analyzed.

The nested PCR-DGGE approach described in this paper has the potential of analysing bifidobacterial communities to the subspecies level. From the methodological point of view, the main advantage of this technique is that a complete analysis of a bifidobacterial community can be performed within a 24 hours time span. Provided that an identification match is obtained with the database, the fact that no further cloning and sequencing of the DGGE bands is necessary makes this technique very suitable for temporal analysis of bifidobacterial ecosystems. Although not yet verified, this approach also holds great promise if applied to other genera, provided that suitable primersets are designed and that the intrageneric taxonomic structure is not too complex.

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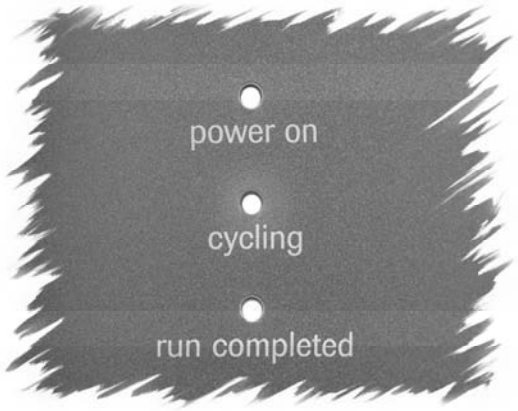
5.3. Real-time PCR Denaturing Gradient Gel Electrophoresis



R. Temmerman, T. Vanhoutte, L. Masco and J. Swings

Summary

The microbial analysis of probiotic products always comprises a qualitative as well as quantitative aspect, the first determining the identity of bacterial species present in a certain product and the latter their total counts. Although a wide array of highly reproducible identification methods for Lactic Acid Bacteria (LAB) exists, the outcome of the analysis mainly relies on the culture media used to isolate the bacteria. As a result, culture-independent methods such as Denaturing Gradient Gel Electrophoresis (DGGE) have been developed to circumvent the limitations of conventional cultivation. However, a remaining drawback of the DGGE approach is that no quantitative information concerning the level of bacterial viability in probiotic products is obtained. This study describes the application of real-time PCR coupled to DGGE for the completely culture-independent analysis of probiotic products on a qualitative and quantitative level. A combination of universal V3 primers with SYBR green chemistry showed to be successful for the total non-specific quantification of species in probiotic products and subsequent DGGE analysis of the amplicons.

Introduction

The importance of microbial analysis of probiotic products has been extensively addressed in previous papers (Holzapfel *et al*., 1998: Hamilton-Miller *et al*., 1999; Temmerman *et al*., 2003a). These analyses usually comprise both a qualitative and a quantitative aspect. Although a wide array of valuable identification methods for Lactic Acid Bacteria (LAB) exists (Temmerman *et al*., 2003c), the outcome of the analysis mainly relies on the use of culture media to isolate the bacteria present in the probiotic product. Because these culture-dependent approaches have proven limitations in terms of recovery rate and reproducibility, the set of recovered isolates may not always truly reflect the microbial composition of the product (Ampe *et al*, 1999; Ercolini *et al*., 2001). For instance, the detection of bifidobacteria in probiotic products impairs the reproducibility of the culture-dependent approach, because of the lack of suitable selective isolation media (Roy, 2001). As a result, culture-independent methods have been developed to circumvent the limitations of conventional cultivation.

A recent study by Temmerman and co-workers (2003b) has demonstrated that Denaturing Gradient Gel Electrophoresis (DGGE) is a fast, reliable and reproducible culture-independent approach for the qualitative analysis of probiotic products and that it has a higher detection and identification potential compared to conventional culture-dependent methods. However, a remaining drawback of the DGGE approach is that no quantitative information concerning the level of bacterial viability in probiotic products is obtained, implying that culture-dependent analysis is still required in order to obtain total counts. As a result, real-time PCR (or quantitative PCR) has been developed to enable the quantification of the initial amount of DNA present in a certain sample, by means of measuring the amount of amplicon generated throughout the PCR reaction. In the field of microbiology, real-time PCR has been performed for the fast culture-independent quantification of pathogenic or inculturable micro-organisms through the combined use of specific primers and intercalating dyes or specific fluorescently labeled probes. This type of specific probes was recently used by Hein and co-workers (2002) in order to detect the possible presence of *Listeria monocytogenes* in milk and other dairy products.

However, the application of a real-time PCR based method for the quality analysis of probiotic products implies that for each possible probiotic species a separate probe or primer set should be available, resulting in an enormous increase of cost and workload. Therefore, it was decided to optimise a real-time PCR approach combining the universal V3 primers with SYBR green chemistry for the total non-specific bacterial quantification in probiotic products. After this quantification step, the amplicons were further analysed using DGGE as described by Temmerman and co-workers (2003b).

Principle of real-time PCR

The ability to monitor the real-time progress of PCR has completely revolutionized the way one approaches PCR- based quantification of DNA and RNA. Real-time PCR now makes quantification of DNA and RNA much more precise and reproducible because it relies on threshold cycle (C_T) values determined during the exponential phase of PCR rather than at the endpoint. By means of fluorophores, either labeled probes or SYBR green (**Fig 1 and 2**) included in the PCR mix, fluorescence is recorded during every cycle and represents the amount of product amplified at that point in the amplification reaction. The more template DNA present at the beginning of the reaction, the fewer number of PCR cycles it takes to reach a point in which the fluorescent signal is first recorded as statistically significant above background, which is the definition of the C_T value (**Fig 3**). From a dilution series of a representative sample, the Colony Forming Units (CFU) value is determined by plate counting and subsequent plotting of the C_T values against these CFU values provides a standard curve applicable for quantification of unknown samples of similar nature. The obtained C_T value of such an unknown sample is compared with the standard curve, from which the corresponding CFU value can be deduced (**Fig 4**).

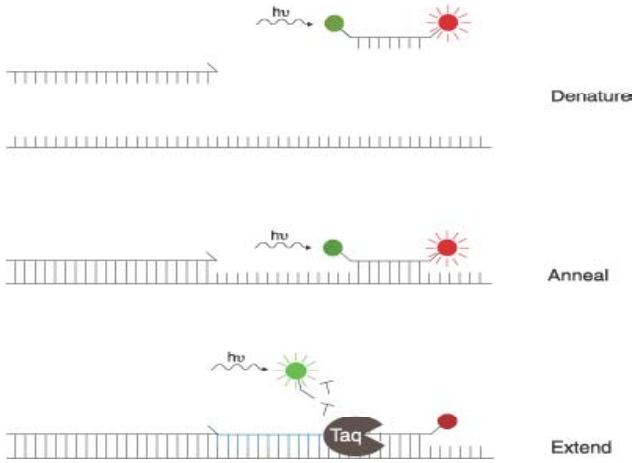


Fig 1. Schematic representation of real-time PCR with TaqMan probes. In the intact TaqMan probe, energy is transferred (FRET process) from the short-wavelength fluorophore on one end (green dot) to the long-wavelength fluorophore on the other end (red dot), quenching the short-wavelength fluorescence. After hybridization, the probe is susceptible to degradation by the endonuclease activity of a processing *Taq* polymerase. Upon degradation, FRET is interrupted, increasing the fluorescence from the short-wavelength fluorophore and decreasing the fluorescence from the long-wavelength fluorophore, resulting in a fluorescent signal detected by the real-time PCR machine.

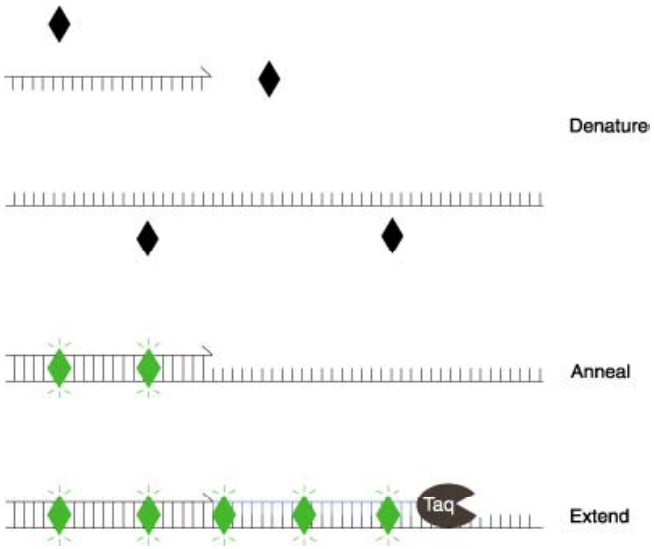


Fig 2. Schematic representation of real-time PCR with the SYBR Green I dye. SYBR Green I dye (black diamonds) becomes fluorescent (green diamonds) upon binding to double-stranded DNA, providing a direct method for quantifying PCR products in real time.

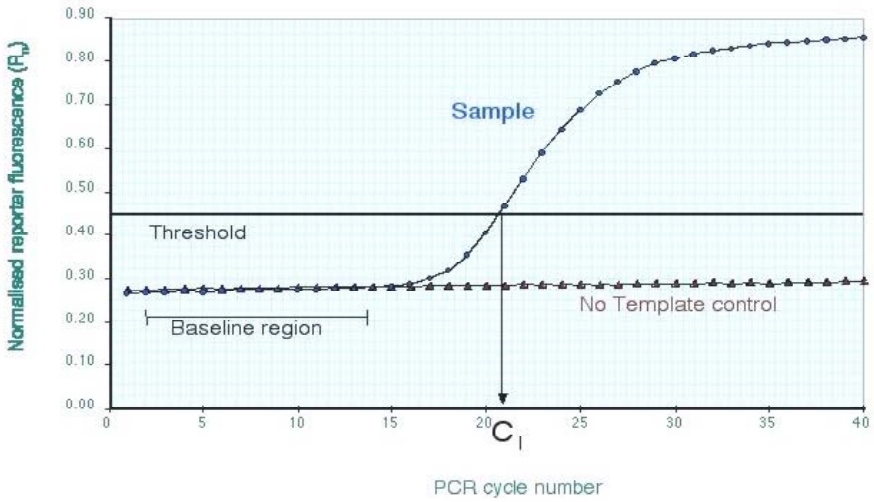


Fig 3. The threshold cycle ($\mathbf{C_T}$) is **the PCR cycle number** for which the fluorescent signal corresponding to the amount of amplified DNA, is first recorded as statistically significant above background.

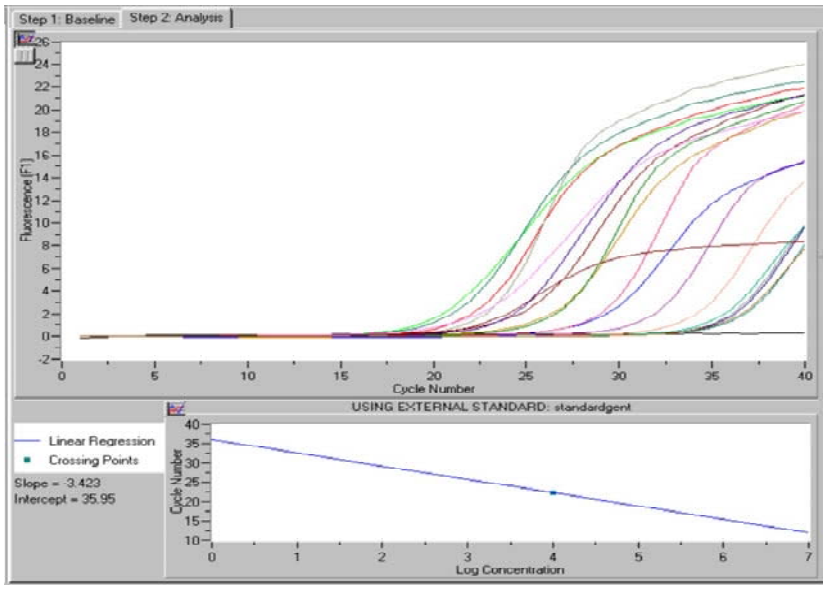


Fig 4. Analysis of a series of bacterial samples. The obtained C_T value for each sample (not indicated on the graph) can be incorporated in the standard curve below (plotting C_T values on the Y-axis against Log concentration values on the X-axis) to determine the corresponding Log concentration value (i.e. CFU/ml).

Material and Methods

Sample and strain collection. The following bacterial strains previously isolated from probiotic products, or originating from the BCCM^{TM}/LMG bacteria collection, were used for the optimisation of real-time PCR: *Lactobacillus casei* (R15648), *Streptococcus thermophilus* (R15652), *Lactococcus lactis* subsp. *lactis* (R15654), *Bifidobacterium lactis* (LMG 18314^{T}) and *Lactobacillus acidophilus* (R15650). Furthermore, the identification potential of the optimised method was verified for the 16 products listed in **Table 1**.

DNA extraction. DNA extraction from pure cultures and probiotic products was based on the protocol by Pitcher and co-workers (1989) with modifications according to Temmerman and colleagues (2003b).

Real-time PCR efficiency. In order to determine the efficiency of amplification using the V3 primers described by Muyzer and co-workers (1993) (F357-GC: 5'-GC-clamp-GCCTACGGAGGCAGCAG-3' and 518-R: 5'-ATTACCGCGGCTGCTGG-3'), 10-fold dilution series of 5 pure cultures (*Lactobacillus casei, Streptococcus thermophilus, Lactococcus lactis* subsp. *lactis, Bifidobacterium lactis* and *Lactobacillus acidophilus*) and of a mixture containing all 5 species were prepared. Dilutions down to 10^{-5} were subjected to DNA extraction and real-time PCR using the following composition of reaction mix per sample: 11.2 µl PCR water, 0.8 µl $MgCl_2$ (final concentration of 2 mM), 2 µl of each primer, 2 µl of template DNA and 2 µl of master mix (containing *Taq* polymerase, SYBR green, dNTP's and PCR-buffer). The PCR programme performed on the Lightcycler^{TM} (Roche Diagnostics, Basel, Switzerland) consisted of a denaturation step (5 min 95°C); amplification step (30 cycles of 15 sec 95°C, 35 sec 55°C, 45 sec 72°C); final elongation step (5 min 72°C) and melt-curve determination (from 60°C to 90°C at a 0.1°C/sec rate). The measuring of the fluorescence was performed at the end of each elongation step (45 sec 72°C) and continuously during the melt-curve determination.

Magnesium titration. The optimal $MgCl_2$-concentration for the PCR reaction mix was determined by amplifying 2 pure cultures (*L. casei* and *B. lactis*) in duplicate using the same PCR programme as described above. $MgCl_2$ concentrations of 2, 3, 4 and 5 mM were tested by means of altering the amounts of $MgCl_2$ and PCR water in the PCR reaction mix.

Standard curve. Before the bacteria in unknown samples can be quantified, it is necessary to generate a standard curve to be included with an external standard in subsequent analysis. Ten-fold dilution series of 2 pure cultures (*L. casei* and *B. lactis*) and one mixture of 5 pure cultures (see above) were plated on MRSA and incubated at 37°C for 72h in order to obtain the CFU/ml value of each dilution. These dilutions were analysed using the real-time protocol described under the determination of the amplification efficiency. The most suitable dilution series was used as a standard curve plotting the CFU/ml on the X-axis (log concentration) and the C_T value on the Y-axis.

Quantification. For the quantification of bacteria in probiotic products the same real-time PCR protocol was used ($MgCl_2$ concentration of 2 mM). Each run included one dilution sample from the series previously used to create a standard curve. The Lightcycler™ software adjusts the standard curve using this calibration point and calculates the CFU/ml on the basis of the C_T value for each sample (quantification using an external standard curve). Using this protocol, 16 probiotic products were analysed.

DGGE. Qualitative analysis of probiotic products was performed using Denaturing Gradient Gel Electrophoresis (DGGE) as previously described by Temmerman and co-workers (2003b). After real-time PCR, the capillary tubes were centrifuged upside-down to collect the amplicon in a PCR tube, from which it could be loaded onto the DGGE gel.

Results and Discussion

A total of 16 probiotic products was subjected to real-time PCR combined with DGGE in order to perform a fast and completely culture-independent quantitative as well as qualitative microbial analysis. A study by Malinen and colleagues (2003) verified the potential of both SYBR and TaqMan chemistry in order to detect the 16S rRNA genes of six bacterial species typically present in human faeces or used in the dairy industry. The authors found that both chemistries tested had an equal sensitivity to detect and quantify bacterial subpopulations present in fecal samples and dairy products. Therefore, we opted for SYBR green, because this allowed the fast and direct quantification of all bacteria in the products without the need for a wide range of (expensive) specific probes. Optimisation of the real-time PCR protocol was performed using a collection of five isolates from probiotic products. Because the amplicons were to be analysed on a DGGE gel, the same V3-primers were used as described by Temmerman and co-workers (2003b). As a result, the conventional PCR protocol for these primers was adopted as the basis for the optimisation of the real-time PCR protocol. Although this produced satisfying amplification products, it was possible to shorten the duration of each step in the PCR cycles because of the capillary tubes allowing much faster heating and cooling of the PCR mixture inside. This 'shortened' PCR program showed good results and was used throughout the rest of the research. An important factor in real-time PCR is the concentration of $MgCl_2$ in the master mix and magnesium-titration indicated 2 mM of $MgCl_2$ to be the most suitable concentration.

The efficiency of amplification using this real-time PCR protocol was determined by analysing 10-fold dilution series of 5 pure cultures and 1 mixture of bacterial species. Both the species in the mixture as well as all separate pure cultures displayed a similar shape of curve in the exponential phase of the PCR (**Fig. 5**). These results indicate that the V3 primers are suitable for use in real-time PCR and no differences in efficiency of amplification occur between different species in a bacterial community. Consequently, it is also possible to create a standard curve based on a certain bacterial pure culture, which can be used for quantification of other bacterial species or mixtures.

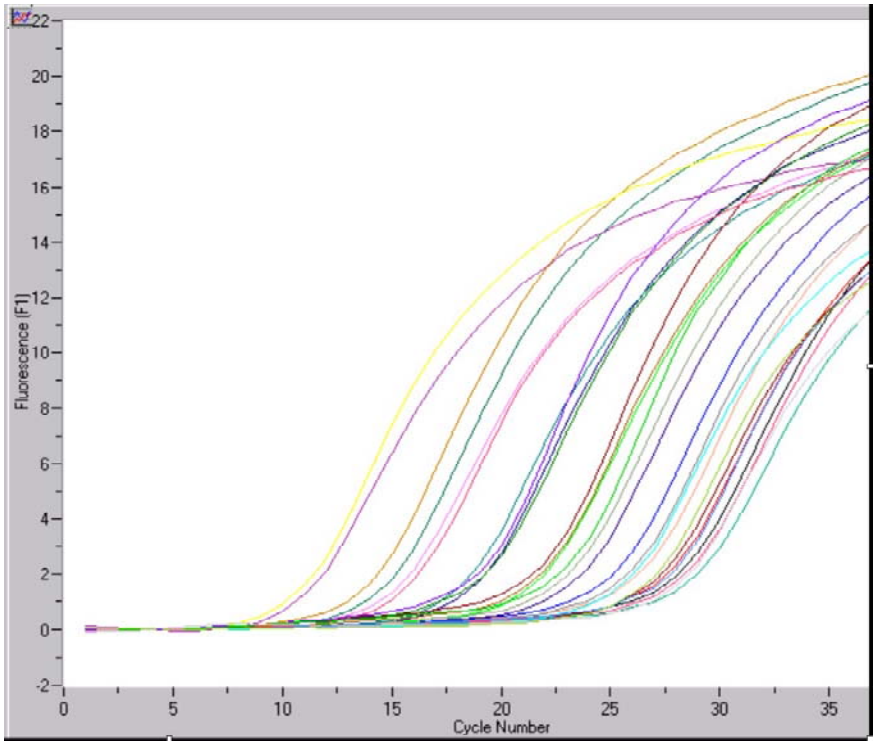


Fig. 5. The efficiency of amplification using the optimised real-time PCR protocol was determined by analysing dilution series of 5 different pure cultures and 1 mixture of these pure cultures. An equal efficiency is obtained when the curves of all samples run parallel to each other during the exponential phase of the PCR.

In some cases, the negative control also produced an amplicon of which melt-curve analysis showed this to be resulting from primer dimers originating from the mutual hybridisation of the GC clamps attached to the primers (Temmerman *et al*., 2003b). In samples, however, primers apparently prefered binding to the present bacterial template DNA, so dimers were not formed. When the number of cycles is too high (e.g. 40), an amplification of the V3 region from *Escherichia coli* may occur, because small amounts of this organism are present in the *Taq* polymerase master mix. However, the PCR protocol used in this study never exceeded 30 cycles and no *E. coli* DNA was amplified in amounts surpassing the background level of fluorescence.

Preceding the microbial quantification of an unknown sample, a standard curve has to be created linking C_T values to CFU/ml or CFU/g. Usually, in real-time PCR, a standard curve is generated from a dilution series constructed from a 'reference' sample. The identity of the reference sample is not important as long as the relevant PCR target is present and the efficiency of amplification is similar for various samples (**Fig 5**). For accurate relative quantification, it is essential that the dilution series, from which the standard curve is generated, is carefully prepared. Subsequently, real-time PCR is performed on several experimental samples and one sample named the 'calibrator', which is one sample from the standard dilution series. For each unknown experimental sample, the obtained C_T values are compared to the standard curve generated from the reference standard, from which the CFU/ml is obtained (**Fig 4**). In our study, a dilution series of *L. acidophilus* was used to create the standard curve (**Fig 6**).

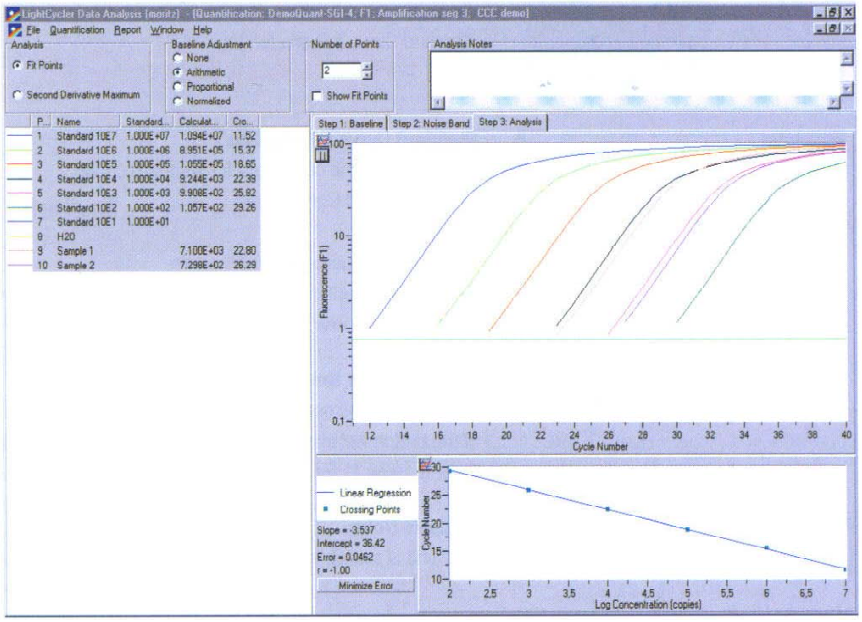


Fig 6. Creation of the standard curve based on a dilution series of *L. acidophilus.* Each 10-fold dilution invoked an increase in C_T value of app. 3.3 cycles indicating that the efficiency of amplification and the dilution series were sufficient for the use of this curve in further quantification of unknown samples.

Table 1 presents the quantification results for 16 products, subjected to conventional culture-dependent analysis, as well as to quantification using real-time PCR. For only five products (ABC plus, Actisun, Bifidus, Yakult, Delhaize-yoghurt) the obtained CFU values from both methods corresponded within one log unit. For six products (Actilus, Actimel, Activia, Bactisubtil, Beneflora and Proflora) the CFU values obtained with real-time PCR were substantially lower than those derived from culture media. This may indicate that the DNA extraction method is not optimal for quantitative extraction of DNA from those products. Since the efficiency of amplification is equal for all products, the PCR itself is most likely not responsible for the poor quantification. From two products (Bacilac and Benefact1), no bacteria were obtained on isolation media, whereas real-time PCR detected high amounts of bacterial cells. This may indicate that real-time PCR also detects DNA amplified from dead bacteria. In the future, the application of Reverse-Transcriptase real-time PCR may cope with this issue, being capable of quantifying only the metabolically active bacteria in the product. Finally, for Benecol, real-time PCR did not produce any fluorescent signal, although high numbers of bacteria were detected using culture media. The analysis of the product using conventional PCR and DGGE identified three bacterial species in the product. The reason why real-time PCR did not detect any bacteria in this product is not clear and merits further research.

Finally, it was verified whether the amplicons resulting from real-time PCR produced the same bands on a DGGE gel, as those obtained after conventional PCR. Identification results are presented in **Table 1** and a DGGE gel showing a comparison of both amplicon types is presented in **Fig. 7**. For all probiotic species encountered during analysis of the 16 products, all amplicons of both PCR techniques coincided on DGGE gels, on the condition that the same primers were used (including GC clamp). The same V3 primers, but without the GC clamp, were also evaluated during real-time PCR in order to completely exclude primer dimer formation. However, the amplicons were found at band positions higher in the DGGE gel, and for some species, the amplicon denatured completely and ran through the gel. These results indicate the need for GC-clamp containing primers (Muyzer *et al*., 1993), although they might produce primer dimers in the negative control sample as demonstrated by melt-curve analysis after real-time PCR.

All together, this study clearly demonstrates that conventional PCR can be replaced by its real-time variant for the microbial analysis of probiotic products. In the near future, optimisation of the DNA extraction should improve the reliability of the quantification for all types of probiotic products. Additionally, implementation of Reverse Transcriptase real-time PCR will allow differentiating between dead and live bacteria.

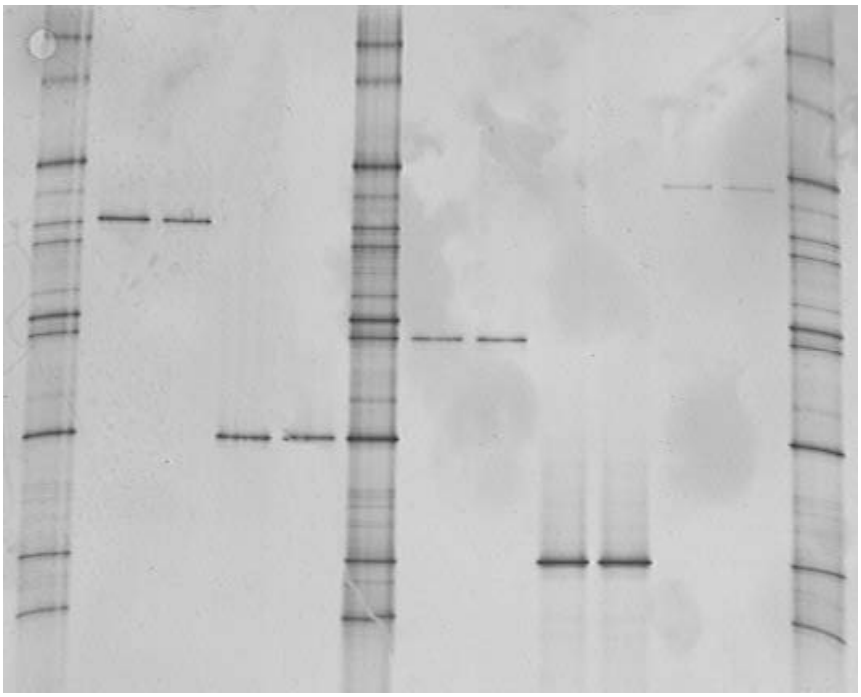


Fig. 7. DGGE gel containing the amplicons of *Lactobacillus acidophilus* (lane 2,3)*, Lactobacillus rhamnosus* (lane 4,5)*, Bacillus cereus* (lane 7,8)*, Bifidobacterium lactis* (lane 9,10) and *Lactobacillus johnsonii* (lane 11,12) obtained after conventional (left lane) and real-time PCR (right lane). Lanes 1, 6 and 13 contain the reference pattern for normalisation of the gel. The gel was stained in SYBR green solution for 30 min.

Table 1. Results of the culture-dependent and culture-independent quantification of DGGE identified bacteria in probiotic products.

Product	Producer	Quantification		Identified using DGGE
		Culture-media	Real-time PCR	
Dairy based products and fruit drinks				
ABC plus	Yomo	1.1E7 CFU/ml	9.57E6 CFU/ml	*L. acidophilus, L. casei, L. delbrueckii* subsp. *bulgaricus, S. thermophilus, B. lactis*
Actilus	Champion	5.9E8 CFU/ml	3.13E5 CFU/ml	*S. thermophilus, L. delbrueckii* subsp. *bulgaricus, B. lactis*
Actimel	Danone	4.5E8 CFU/ml	9.63E4 CFU/ml	*L. casei, S. thermophilus, L. delbrueckii* subsp. *bulgaricus*
Actisun	Triballat Noyal	1.4E7 CFU/ml	6.86E7 CFU/ml	*S. thermophilus, L. delbrueckii* subsp. *bulgaricus, L. acidophilus, L. casei*
Activia	Danone	5.9E8 CFU/ml	5.16E7 CFU/ml	*S. thermophilus, L. delbrueckii* subsp. *bulgaricus, Lc. lactis, B. lactis*
Benecol	Janssen-Cilag	9.5E8 CFU/ml	No signal	*S. thermophilus, L. acidophilus, B. lactis*
Bifidus	CABAC	7.9E8 CFU/ml	1.16E8 CFU/ml	*S. thermophilus, L. delbrueckii* subsp. *bulgaricus, B. lactis*
Yakult	Yakult	1.4E8 CFU/ml	1.88E8 CFU/ml	*L. casei*
Yoghurt	Delhaize	9.6E8 CFU/ml	1.08E9 CFU/ml	*S. thermophilus, L. delbrueckii* subsp. *bulgaricus, B. lactis*

Table 1 (Continued). Results of the culture-dependent and culture-independent quantification of DGGE identified bacteria in probiotic products.

Product	Producer	Quantification		Identified using DGGE
		Culture-media	Real-time PCR	
Freeze-dried products				
Bacilac	THT	0 CFU/g	1.10E7 CFU/g	*L. rhamnosus, L. helveticus*
Bactisubtil	Synthelabo	1.6E9 CFU/g	5.12E7 CFU/g	*Bacillus cereus*
Benefact1	Reformwaren	0 CFU/g	1.85E9 CFU/g	*S. thermophilus, L. delbrueckii* subsp. *bulgaricus*
Beneflora	ORTIS	1.7E7 CFU/g	9.68E4 CFU/g	*L. acidophilus, B. lactis, S. thermophilum, B. longum, L. delbrueckii* subsp. *bulgaricus, L. casei*
Decoflor	Decola	4.6E7 CFU/g	2.89E9 CFU/g	*L. acidophilus, L. delbrueckii* subsp. *bulgaricus, L. rhamnosus, S. thermophilus*
Hygiaflora	OCE BIO	7.7E6 CFU/g	5.22E8 CFU/g	*L. casei, S. thermophilus, L. delbrueckii* subsp. *bulgaricus, B. breve, L. acidophilus*
Proflora	Chefaro	1.3E10 CFU/g	9.65E4 CFU/g	*L. acidophilus, L. delbrueckii* subsp. *bulgaricus, B. lactis, S. thermophilus*

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Part 3

Conclusions and Perspectives

Conclusions and Perspectives

The research performed during this PhD work has contributed to the microbial quality control of commercial probiotic products, an aspect of probiotics that has often been neglected in the past. Essentially, both quantitative and qualitative microbial analysis of products was performed, as well as a screening of probiotic isolates for their antibiotic susceptibility and their potential to survive passage through the GI-tract. Culture-dependent and culture-independent microbial analysis of probiotic products revealed that a substantial number of freeze-dried products and - to a lesser extent - dairy products was incorrectly or inadequately labeled concerning the total count and identity of the incorporated probiotic strains. Using the culture-dependent approach, mainly bifidobacterial species were hard to recover, and besides the total absence of these organisms in many products, this may be due to the applied method of detection. Although still far from optimal, improved protocols and selective media with a higher performance have recently been developed, for enumeration of bifidobacteria from (probiotic) samples. Furthermore, from a substantial percentage of products no strains could be isolated, indicating that bacteria are either absent or are present in numbers beneath 10^3 CFU/ml-g, considering the detection limit of culture-dependent analysis. Consequently, it can be concluded that these products are unlikely to produce any probiotic effect, when bacterial numbers are well below the desired minimum dose of 10^6 CFU/ml-g.

In order to circumvent the possible pitfalls of culture-dependent analysis, it was a major aim of this work to optimise the Denaturing Gradient Gel Electrophoresis (DGGE) technique as a culture-independent method for qualitative microbial analysis. In comparison, this technique can overcome some of the major disadvantages or limitations of conventional culture-dependent analysis, in terms of speed, detection level, taxonomic resolution and reproducibility. By means of digital capturing and normalisation of the DGGE gel patterns, the direct identification of the amplicons avoided the use of labour-intensive cloning and sequencing. For all probiotic species, unique band positions were obtained, which allowed a fast direct identification. Nevertheless, some of these band positions may coincide with these of other organisms such as non-probiotic LAB, possible contaminants or food pathogens, after further extensions of the identification database. In future research, these situations can be avoided by the application of species- or group-specific primers, the use of narrow gradient gels or the adjustment of other DGGE parameters. Sequencing of specific DGGE bands can therefore

not be excluded completely, in case all former modifications to the original setup do not solve the problems. Furthermore, the design of a well-distributed reference pattern is crucial, because an optimal normalisation is needed in order to reduce the possible errors in establishing band positions. These minor inter-gel differences were found to be important in the distinction of closely related species, such as those of the *L. acidophilus* group, which could only be separated using a narrow gradient DGGE-gel. A further optimisation of the DGGE method combining multiple amplicons demonstrated that this method is suitable for the species-specific identification of bifidobacterial species present in various ecosystems. However, because quantification still requires the use of culture media, it was investigated whether the conventional PCR step in the DGGE approach can be replaced by real-time PCR, in order to allow the complete culture-independent analysis of bacteria in probiotic products. Nevertheless, culture-dependent analysis is still necessary to investigate other properties of the probiotic strains such as antibiotic resistance and GI-tract survival. It was demonstrated that a rather high number of isolates from probiotic products displayed phenotypic antibiotic resistance against kanamycin, tetracycline, chloramphenicol and/or erythromycin. The assessment of the ability to survive passage through the GI-tract, as well as the hydrophobic nature showed these characteristics to be species- or even strain-specific.

Although the concept of probiotics originates from decades ago, research performed in this field has witnessed an exponential increase during the past few years, triggered by the growing importance of healthy nutrition in our society. Most of this research has focussed on the demonstration of health promoting effects by selected probiotic strains. Ideally, *in vivo* studies of probiotic effects are performed according to the double-blind placebo-controlled crossover concept. Current research is also shifting towards molecular and biochemical work in order to unravel the underlying mechanisms of these health effects. So far, the attention paid to safety and quality control of probiotic strains and products is almost negligible. A few papers have highlighted the importance of this type of studies. In the framework of the European PROEUHEALTH cluster (http://www.vtt.fi/virtual/proeuhealth/), one out of 8 research projects is dedicated to assess the biosafety of probiotic bacteria for human consumption, i.e. the PROSAFE project (http://lmg.UGent.be/prosafe/).

The work performed in this thesis has clearly demonstrated the need for microbial analysis of probiotic products. The optimised DGGE method resulting from this research allows a fast product quality control at various stages of the production process, which makes the technique highly applicable for the industry. In this context, the further optimisation of the real-

time PCR technology is of major importance. Although the concept of culture-independent quantitative microbial analysis has been clearly demonstrated in this thesis, the reproducibility of the method has to be further evaluated. Such an evaluation will certainly include optimisation of the DNA extraction protocol, which is essential in the interpretation of real-time PCR data. The search for a range of genus-, species- or even strain-specific probes is expected to improve the detection limit and the reliability of quantification. This will also allow qualitative analysis in such a way that DGGE analysis of the PCR amplicons may become redundant for samples with a rather simple microbial composition. On the other hand, further optimization is of crucial importance to develop a universally applicable method, which allows a fast, reproducible, reliable and complete microbial analysis of probiotic products. Evaluation of other molecular techniques, such as micro-array technology, is also expected to contribute to a reliable quality control of (probiotic) products and samples. Finally, on the taxonomic level, the possible influence of operon heterogeneity and strain-to-strain differences on the stability of the DGGE patterns should be investigated by means of other genotypic methods, in order to gain a better insight in the underlying phylogenetic structure of certain heterogeneous groups of bacteria.

Although specific antibiotic resistance traits among probiotic strains may be desirable, the finding of atypical resistance in probiotic isolates indicates that continuous attention should be paid to the selection of probiotic strains free of transferable antibiotic resistance. Because of the magnitude of such research, this issue was not addressed any further in the scope of this thesis. Expert research groups such as those involved in the 'PROSAFE' project are currently investigating the presence of antibiotic resistance determinants in probiotic strains. Meanwhile, new and more optimised protocols for the determination of antibiotic susceptibility in anaerobic bacteria have been developed, and should be further assessed in the near future. In addition to this safety aspect, the ability of probiotic strains to survive passage through the GI-tract in order to reach the large intestine is an important issue of functionality. As demonstrated in this thesis, such properties appear to be species- or even strain-specific. Fluctuations and seemingly contradictory results (e.g. in case of the hydrophobicity testing) may be due to a wide number of parameters, such as the inconsistency of the bacterial cell wall, the presence of multiple clones in a pure culture, the influence of temperature, etc… Therefore, in order to further elaborate on the conclusions from *in vitro* tests, *in vivo* studies are needed to determine their correspondence to real-life situations. Depending on the outcome of the ongoing discussion about whether dead bacteria may exert probiotic effects, the importance of GI-tract survival will either be enforced or weakened. An even greater

disagreement exists about the need of colonisation or adhesion of probiotic strains in a certain region of the GI-tract. However, demonstration of these properties by means of *in vivo* trials remains very difficult because of the lack of healthy human volunteers.

The development of a successful probiotic product includes many aspects of safety, functionality as well as technological and labeling issues. All together, the results obtained in the course of this work demonstrate the need for a profound microbial analysis of probiotic products, in which DGGE has demonstrated its high competence. It is of paramount importance that in an era during which consumers become more aware of the importance of functional nutrition and health, probiotic products are safe and well documented in order to provide consumers with all beneficial aspects of probiotics. Mainly because of the lack of legislation, profound quality control is currently lacking. Clearly, Working Committees and Discussion Groups of the EC and the FAO/WHO should take the lead in establishing a sound scientific basis for a broadly acceptable legislation on the safety and quality of probiotics.

Summary

In order to develop a successful probiotic product with long-term marketing potential, pre-production research towards safety and functional properties of the included probiotic strains has to be performed, as well as an efficient quality control of the product itself. **The goal of this PhD work was to evaluate and optimise new and existing methodologies to examine the microbial aspects of probiotic product quality control**. Essentially, quantitative and qualitative microbial analysis of products was performed, as well as screening probiotic isolates for their antibiotic susceptibility and potential to survive passage through the Gastro Intestinal (GI)-tract.

In a first study, **culture-dependent analysis** of 55 European probiotic products was performed with regard to total bacterial counts, as well as the identity and antibiotic resistance of the recovered isolates (Temmerman *et al.*, 2003a). Using a range of elective culture media, a total of 268 bacterial isolates was obtained from 30 dried food supplements and 25 dairy products. Bacterial recovery was obtained from 63% of the dried food supplements, with total counts ranging from 10^3 to 10^6 CFU/g. In contrast, all dairy products yielded growth in the range of 10^5 to 10^9 CFU/ml. Mainly bifidobacterial species were hard to recover, and besides the total absence of these organisms in many products, this may be due to the applied method of detection; for instance no reliable medium for enumeration of these organisms exists. For each product, the microbial label information was checked through taxonomic characterization of the recovered isolates using whole-cell protein profiling. Mislabeling was noted in 47% and 40% of the food supplements and dairy products, respectively. In 19 products, the isolated species were entirely different from those mentioned on the product label. For instance, *Enterococcus faecium* was isolated from six food supplements whereas only two of those products actually claim this species on their label.

Using the disc diffusion method, **antibiotic resistance** among 187 isolates was detected against kanamycin (79% of the isolates), vancomycin (65%), tetracycline (26%), penicillinG (23%), erythromycin (16%) and chloramphenicol (11%). Overall, 68.4% of the isolates showed resistance against multiple antibiotics, including intrinsic resistances. Furthermore, 38% of the *E. faecium* isolates displayed vancomycin resistance, which was disproved by additional phenotypic and PCR assays. Although specific antibiotic resistance

traits among probiotic strains may be desirable, the finding of atypical resistance in probiotic isolates indicates that continuous attention should be paid to the selection of probiotic strains free of transferable antibiotic resistance. Because of the magnitude of such research, this issue was not addressed any further in the scope of this thesis. Expert research groups (http://lmg.UGent.be/prosafe/) are currently investigating the presence of antibiotic resistance determinants in probiotic strains.

In addition to this safety aspect, the ability of probiotic strains to survive **passage through the GI-tract** in order to reach the large intestine is an important issue of functionality. Probiotic strains must tolerate the acidic and protease-rich conditions of the stomach, and survive and grow in the presence of bile acids. A series of *in vitro* tests was applied to screen 18 probiotic isolates, representing 12 species, for their resistance against pepsin, low pH and pancreatin, as well as for their growth performance in the presence of bile salts. Furthermore, using 5 solvents, the hydrophobic nature of the isolates was determined as an indication of their adhesion potential. *L. crispatus*, *L. reuteri* and both *L. johnsonii* isolates were shown to be highly resistant against an acidic pepsin-containing solution, with *L. reuteri* showing slight bacterial growth. Although the remaining probiotic isolates demonstrated less survival capacity, they scored significantly better than the two starter cultures *L. delbrueckii* subsp. *bulgaricus and S. thermophilus,* being very susceptible to low pH. Except for both *L. casei* isolates, all strains showed high resistance against pancreatin, for which survival capacity was tested in a more neutral solution of pH 8. Classification of the isolates according to their resistance against bile acids was determined as the difference in time needed for bacterial suspensions to reach an optical density of 0.3 in the presence or absence of bile acids. Six isolates were resistant to bile acids, seven were tolerant and five were sensitive. As expected, both *L. delbrueckii* subsp. *bulgaricus and S. thermophilus* were sensitive. In combination with their very low potential for gastric survival these species are unlikely to reach the large intestine alive. Finally, the hydrophobic nature of the isolates was assessed as a measure for potential adhesion, and again substantial strain differences were noticed. Fluctuations and seemingly contradictory results may be due to a wide number of parameters, such as the inconsistency of the bacterial cell wall, the presence of multiple clones in a pure culture, the influence of temperature, etc. Therefore, in order to further elaborate on the conclusions from *in vitro* tests, *in vivo* studies are needed to determine their correspondence to real-life situations.

In order to circumvent the possible pitfalls of culture-dependent analysis, it was a major aim of this work to optimise the **Denaturing Gradient Gel Electrophoresis (DGGE)** technique as a **culture-independent method** for qualitative microbial analysis. In order to validate the DGGE approach, a collection of ten probiotic products, including four dairy products, one fruit drink and five freeze-dried products, was screened by means of parallel DGGE and culture-dependent analysis (Temmerman *et al*., 2003b). The culture-independent DGGE approach involved extraction of total bacterial DNA directly from the product, PCR amplification of the 16S rDNA - V3 region, and separation of the amplicons on a DGGE gel. Identification was performed after normalisation of the gel pattern using a standard reference pattern, followed by comparison of the band positions with those of well-characterized type and reference strains present in a new user-generated BioNumerics database. As determined earlier, culture-dependent analysis revealed colony counts that were substantially lower in case of the freeze-dried products, yielding counts between 10^5 and 10^7 CFU/g of product, compared to the dairy products producing numbers between 10^7 and 10^9 CFU/ml. Furthermore, 6 products demonstrated mislabeling in terms of bacterial identity. A comparison of these results with those of the culture-independent DGGE analysis produced two different scenarios. In case of 5 products both methods produced the same results, whereas in case of the 5 remaining products DGGE analysis was able to detect more species than those recovered by isolation. In general, this study clearly demonstrated DGGE to overcome some of the major disadvantages or limitations of conventional culture-dependent analysis, in terms of speed, detection level, taxonomic resolution and reproducibility.

The identification to the species level of **bacterial communities** is difficult to combine with the monitoring of its temporal changes. Most identification methods are not designed to visualise entire microbial communities, whereas techniques developed for the analysis of bacterial ecosystems generally exhibit a poor or labour-intensive identification potential. Previously, it was demonstrated that DGGE is suitable for the analysis of probiotic products, each of which represent a bacterial community with a rather low taxonomic complexity. The following study focussed on more complex communities and involved the optimisation and validation of a nested PCR-DGGE approach for the species-specific analysis of bifidobacterial communities (Temmerman *et al*., 2003c). The method comprises a *Bifidobacterium*-specific PCR, followed by purification of the amplicons, which serve as template DNA for the second PCR step amplifying the V3 and V6-V8 region of the 16S rDNA. Both amplicons are analysed on a DGGE gel, after which the combined band positions are compared with a previously constructed database of *Bifidobacterium* reference strains. The method was validated by means

of four artificial mixtures mimicking the possible bifidobacterial flora of the human and chicken intestine, rumen and sewage, and by means of two faecal samples. Except for *B. coryneforme* and *B. indicum*, all bifidobacteria originating from various ecosystems could be identified in a highly reproducible manner. Because no further cloning and sequencing of the DGGE bands is necessary, this nested PCR-DGGE technique can be completed within a 24 hours span. Furthermore, it shows great promise for the species-specific monitoring of temporal changes in bifidobacterial communities, such as tracking probiotic bifidobacteria in faecal samples.

The importance of DGGE for culture-independent analysis of probiotic products on the qualitative level was demonstrated to be very accurate, although quantification still requires the use of culture media. In this context, the final study performed in the course of this PhD work involved the coupling of **real-time PCR** to DGGE, in order to obtain a fast and completely culture-independent approach of quantitative as well as qualitative microbial analysis of 16 probiotic products. Real-time PCR has been developed to determine the initial amount of DNA present in a sample by means of measuring the formation of amplicon throughout the PCR reaction. In practice, the application of this method for the quality analysis of probiotic products would imply that for each possible probiotic species a separate probe or primer set should be used, which will significantly increase cost and workload. Therefore, it was decided to optimize a real-time PCR approach combining the universal V3 primers with SYBR green chemistry for the total non-specific quantification of species in probiotic products. After this quantification step, the amplicons were further analyzed using DGGE. The study pointed out that the applied protocol resulted in a successful and reproducible PCR, with equal amplification efficiencies for all tested pure cultures and products. Subsequent DGGE analysis showed perfect correlation between the amplicons resulting from real-time PCR, compared to conventional PCR. However, the actual quantification seemed unreliable compared to the results obtained using culture media, most probably due to the applicability of the DNA extraction method on different types of products. Although the research in this PhD has clearly demonstrated the possibility to replace conventional PCR by its real-time version and to couple it to DGGE, an extensive optimization of the procedure including the DNA extraction protocol has to be performed.

The development of a successful probiotic product includes many aspects of safety, functionality as well as technological and labeling issues. Generally speaking, the results obtained in the course of this work demonstrate the need for a profound microbial analysis of probiotic products, in which DGGE has demonstrated its high competence. It is of paramount importance that in an era in which consumers become more aware of the importance of functional nutrition and health, probiotic products are safe and well documented in order to provide consumers with all beneficial aspects of probiotics. Mainly because of the absence of legislation, profound quality control is currently lacking. Clearly, Working Committees and Discussion Groups of the EC and the FAO/WHO should take the lead in establishing a sound scientific basis for a broadly acceptable legislation on the safety and quality of probiotics.

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Samenvatting

Voorafgaand aan de succesvolle commercialisering van een probiotisch product is onderzoek naar de veiligheid en functionaliteit van de gebruikte probiotische stammen een noodzaak, naast een efficiënte kwaliteitscontrole van het product zelf. **Dit doctoraat had tot doel een evaluatie en optimalisatie uit te voeren van nieuwe en bestaande technieken voor microbiële kwaliteitscontrole van probiotische producten.** Zowel kweekafhankelijke als kweekonafhankelijke analyses van producten werden uitgevoerd, alsook het screenen van probiotische isolaten (melkzuurbacteriën) op de aanwezigheid van antibiotica-resistenties en naar hun capaciteit om de maag-darm transit te overleven.

Een eerste studie omvatte de **kweekafhankelijke analyse** van 55 Europese probiotische producten, ter bepaling van het totaal aantal aanwezige bacteriën, alsmede hun identiteit en antibiotica resistentieprofielen (Temmerman *et al.*, 2003a). Door gebruik te maken van een reeks electieve groeimedia werden 268 isolaten bekomen uit 30 gedroogde preparaten en 25 zuivelproducten. In 63% van de gedroogde voedingssupplementen werden levende bacteriën teruggevonden, met aantallen van 10^3 tot 10^6 CFU/g. In schril contrast hiermee bleken alle geteste zuivelproducten wel degelijk levende bacteriën te bevatten in concentraties van 10^5 tot 10^9 CFU/ml. Voornamelijk de detectie van bifidobacteriën bleek moeilijk, hetgeen naast hun daadwerkelijke afwezigheid ook te wijten kan zijn aan de gebruikte analysemethode zoals bvb het gebrek aan een betrouwbaar medium voor de telling van deze organismen. Voor elk product werd de label informatie betreffende de identiteit van de aanwezige bacteriën nagegaan door middel van taxonomische karakterisering met eiwitprofilering van de bekomen isolaten. Een incorrect label werd vastgesteld bij 47% van de gedroogde preparaten en 40% van de zuivelproducten. Bij 19 producten bleken de isolaten totaal verschillend te zijn van de geclaimde species. Uit zes gedroogde voedingssupplementen werd bijvoorbeeld *Enterococcus faecium* geïsoleerd, terwijl slechts twee van deze producten dit species vermelden op hun label.

Met behulp van de disk diffusie methode werd de **antibioticagevoeligheid** van 187 isolaten nagegaan. Hierbij werd antibiotica resistentie aangetroffen tegen kanamycine (79% van de isolaten), vancomycine (65%), tetracycline (26%), penicillineG (23%), erythromycine (16%) en chloramphenicol (11%). In totaal bleken 68.4% van de isolaten resistent te zijn tegen meerdere antibiotica, intrinsieke resistenties inclusief. Initieel bleek 38% van de *E. faecium* isolaten resistentie te vertonen tegen vancomycine, hetgeen weerlegd werd met aanvullende

fenotypische en PCR tests. Hoewel een beperkte vorm van antibioticaresistentie bij probiotische stammen voordelig kan zijn, wijst het vinden van atypische resistentieprofielen bij de isolaten op de noodzaak van grondige moleculaire analyse naar de transfereerbaarheid van dergelijke resistenties. Omwille van de omvang van dergelijk onderzoek werd er in het kader van deze thesis geen verder gevolg aan gegeven, temeer er momenteel gespecialiseerde onderzoeksgroepen (http://lmg.Ugent.be/prosafe/) bezig zijn met het opsporen van antibioticaresistentie determinanten in probiotische stammen.

Naast het veiligheidsaspect, betekent de mogelijkheid van probiotische stammen om de **passage naar de dikke darm** te overleven een belangrijk onderdeel van hun functionaliteit. Probiotische bacteriën moeten voldoende tolerant zijn tegen de zure en protease-rijke omgeving van de maag, alsook tegen de aanwezigheid van galzuren. Met behulp van een reeks *in vitro* testen werd de resistentie van 18 probiotische isolaten tegen pepsine, lage pH, pancreatine en galzuren bepaald. Bovendien werd aan de hand van 5 solventen het hydrofobe karakter van de isolaten bepaald als indicatie voor mogelijk adhesiepotentieel. *L. crispatus*, *L. reuteri* en beide *L. johnsonii* isolaten bleken sterk resistent te zijn tegen een zure pepsine-oplossing, waarbij *L. reuteri* zelfs lichte groei vertoonde. Hoewel de andere isolaten minder resistent bleken te zijn, scoorden ze merkelijk beter dan de starterculturen *L. delbrueckii* subsp. *bulgaricus* en *S. thermophilus* die uiterst gevoelig bleken te zijn voor een lage pH. Met uitzondering van beide *L. casei* isolaten vertoonden alle stammen een hoge resistentie tegen het enzym pancreatine bij pH 8. Klassificatie van de isolaten naar hun gevoeligheid voor galzuren is gebaseerd op het tijdsverschil voor twee bacteriesuspensies, respectievelijk met en zonder galzuren, om een optische densiteit van 0.3 te bereiken. Zes isolaten bleken resistent te zijn, zeven isolaten tolerant en vijf isolaten gevoelig. Zoals verwacht waren de starterculturen *L. delbrueckii* subsp. *bulgaricus* en *S. thermophilus* gevoelig, hetgeen in combinatie met hun zeer lage overleving van de maag als gevolg heeft dat deze species de dikke darm normaal niet levend bereiken. Tenslotte werd de hydrofobiciteit van de bacteriële celwand bepaald als maat voor het mogelijke adhesiepotentieel van de stam. Zoals het geval was bij de vorige *in vitro* testen, bleken de resultaten ook hier sterk stamafhankelijk. Schommelingen en schijnbaar tegenstrijdige resultaten kunnen het gevolg zijn van een aantal parameters zoals de ongelijke samenstelling van de bacteriële celwand, de aanwezigheid van meerdere clones in een reinkultuur, de invloed van de temperatuur, enz.. Alvorens hieruit conclusies te trekken is het daarom noodzakelijk dat *in vivo* tests worden uitgevoerd om de verwantschap van *in vitro* tests met reële situaties te bepalen.

Om de mogelijke nadelen van kweekafhankelijke analyse te omzeilen was het voornaamste doel van dit doctoraatswerk de optimalisatie van de Denaturerende Gradient Gel Electroforese (DGGE) techniek voor de **kweekonafhankelijke analyse** van probiotische producten. Om deze DGGE aanpak te valideren werd een vergelijkende kweekafhankelijke en kweekonafhankelijke analyse uitgevoerd van tien probiotische producten, bestaande uit vier zuivelproducten, één vruchtendrank en vijf gedroogde preparaten (Temmerman *et al.*, 2003b). De kweekonafhankelijke DGGE methode omvat extractie van totaal bacterieel DNA rechtstreeks uit het product, gevolgd door een PCR amplificatie van de V3 regio van het 16S rDNA en elektroforetische scheiding van deze amplicons op een DGGE gel. Identificatie van de bacteriën gebeurt via normalisatie van de gedigitaliseerde gelpatronen aan de hand van een standaard referentiepatroon, gevolgd door vergelijking van de bandposities op het gel met deze van de referentiestammen van een nieuw opgebouwde BioNumerics databank. Zoals voorheen toonde de kweekafhankelijke analyse ook nu aan dat het aantal bacteriën in de gedroogde preparaten met waarden tussen 10^5 en 10^7 CFU/g aanzienlijk lager lag dan voor de zuivelproducten met waarden variërend van 10^7 tot 10^9 CFU/ml. Bovendien bleken zes producten verkeerde label informatie te bevatten wat betreft hun bacteriële samenstelling. Vergelijking van deze resultaten met deze bekomen na kweekonafhankelijke analyse via DGGE leverde twee situaties op. Voor vijf producten leverden beide methoden dezelfde resultaten, terwijl voor de andere vijf producten DGGE in staat was om meer species te detecteren dan de kweekafhankelijke methode. Deze studie toonde bijgevolg duidelijk aan dat de DGGE methode sneller, betrouwbaarder en reproduceerbaarder is voor de kwalitatieve microbiële analyse van probiotische producten, met een hoger detectie- en identificatiepotentieel dan de kweekafhankelijke analyse.

De bepaling van de speciessamentelling van **bacteriële gemeenschappen** is moeilijk te combineren met de opvolging van daarin optredende veranderingen in de tijd. De meeste identificatietechnieken zijn niet ontworpen om een complete gemeenschap te visualiseren, terwijl technieken voor de analyse van bacteriële ecosystemen gewoonlijk een laag of arbeidsintensief identificatiepotentieel bezitten. Er werd reeds aangetoond dat DGGE zeer geschikt is voor de analyse van probiotische producten, die in feite kunnen beschouwd worden als eerder eenvoudige microbiële gemeenschappen. Een volgende studie richtte zich op meer complexe gemeenschappen en omvatte de optimalisatie en validatie van een genestelde PCR-DGGE aanpak voor de species-specifieke analyse van bifidobacteriële gemeenschappen (Temmerman *et al.*, 2003c). De methode omvat een *Bifidobacterium*-specifieke PCR stap, gevolgd door de zuivering van de bekomen amplicons, die op hun beurt dienen als template

DNA tijdens een tweede PCR reactie die de amplificatie beoogt van zowel de V3 als V6-V8 regio van het 16S rDNA. Beide amplicons worden geanalyseerd op een DGGE gel, waarna de bandposities worden vergeleken met deze van een voordien opgebouwde databank van *Bifidobacterium* referentiestammen. De methode werd gevalideerd aan de hand van vier kunstmatige mengsels van reinculturen die de mogelijke bifidobacteriële flora nabootsten van het colon van resp. de mens en de kip, het rumen en afvalwater, alsook aan de hand van twee fecale stalen. Met uitzondering van *B. coryneforme* en *B. indicum* bleek de techniek in staat te zijn alle bifidobacteriën te detecteren in verschillende ecosystemen. Aangezien geen verdere clonering en sequenering van de DGGE banden noodzakelijk is, kan deze genestelde PCR-DGGE methode worden uitgevoerd binnen een tijdspanne van 24 uur. Bovendien vertoont de techniek goede capaciteiten om species-specifieke analyses uit te voeren van bifidobacteriële gemeenschappen zoals de opvolging van probiotische stammen in fecale stalen.

De DGGE techniek bleek na optimalisatie zeer accuraat te zijn voor de kweek-onafhankelijke analyse van probiotische producten op kwalitatief niveau, hoewel een kwantificatie van het aantal bacteriën in de producten nog steeds kweekafhankelijk diende te gebeuren. Bijgevolg omvatte de laatste studie in het kader van dit doctoraatswerk de koppeling van real-time PCR aan DGGE, om zodoende een snelle en volledig kweekonafhankelijke kwantitatieve en kwalitatieve analyse te bekomen van zestien probiotische producten. Real-time PCR werd ontwikkeld om de initiële hoeveelheid DNA in een staal te bepalen, door tijdens de PCR reactie de hoeveelheid gevormd amplicon te meten. De toepassing van de methode voor de analyse van probiotische producten zou met zich meebrengen dat voor elk mogelijk probiotisch species een aparte probe of primerset ontwikkeld dient te worden, hetgeen de kostprijs en arbeidsintensiteit sterk zou opdrijven. Daarom werd besloten om real-time PCR te optimaliseren met een combinatie van universele V3 primers en SYBR green detectie, om zodoende een niet-specifieke kwantificatie van alle bacteriën in een product te bekomen. Na deze PCR-stap worden de amplicons geanalyseerd met behulp van DGGE. De studie wees uit dat deze methode resulteerde in een goede en reproduceerbare PCR, met hoge efficiëntie van amplificatie voor alle geteste reinculturen en producten. De DGGE analyse van de amplicons toonde een perfecte overeenkomst aan tussen de amplicons van real-time PCR en deze van conventionele PCR. De eigenlijke kwantificatie van het aantal bacteriën in de producten bleek echter niet betrouwbaar, waarschijnlijk door de DNA extractie methode die geen gelijke efficiëntie oplevert voor alle types van probiotische producten. Hoewel het werk van dit doctoraat duidelijk aantoont dat de vervanging van conventionele PCR door real-time PCR en de koppeling ervan aan DGGE perfect mogelijk is, dient in de onmiddellijke toekomst een grondige optimalisatie van voornamelijk de DNA extractie te worden uitgevoerd.

De ontwikkeling van een succesvol probiotisch product omvat verscheidene aspecten van veiligheid, functionaliteit, technologie en etikettering. De resultaten bekomen in het kader van dit doctoraatswerk tonen duidelijk de nood aan van een grondige microbiële analyse van probiotische producten, waarbij DGGE zeer geschikt bleek. Het is van primordiaal belang dat in een tijd waarin de consument bewust wordt van het belang van gezonde voeding, probiotische producten veilig en goed gedocumenteerd zijn om deze consument te laten genieten van alle gezondheidsvoordelen van probiotica. Voornamelijk omwille van het gebrek aan wetgeving is er een aanzienlijk tekort aan kwaliteitscontrole. Werk- en discussiegroepen van de EC en FAO/WHO zouden een leidende positie moeten innemen in het bekomen van een stevige wetenschappelijke basis voor het opstellen van een algemeen aanvaardbare wetgeving over de veiligheid en kwaliteit van probiotica.

· **Temmerman, R., Pot, B., Huys, G., Swings, J.** (2003a). Identification and antibiotic susceptibility of bacterial isolates from probiotic products. *International Journal of Food Microbiology*. **81(1):**1-10.

· **Temmerman, R., Scheirlinck, I., Huys, G. and Swings, J.** (2003b). Culture-independent Analysis of Probiotic Products using Denaturing Gradient Gel Electrophoresis (DGGE). *Applied and Environmental Microbiology* **69(1):**220-226.

· **Temmerman, R., Masco, L., Vanhoutte, T., Huys, G. and Swings, J.** (2003c). Development and Validation of a Nested PCR- Denaturing Gradient Gel Electrophoresis Method for Taxonomic Characterization of Bifidobacterial Communities. *Applied and Environmental Microbiology.* **In press.**

Appendix and Curriculum Vitae

Appendix - Lists of strains used in this study

Table 1: List of probiotic isolates, deposited in the Research collection of the Laboratory of Microbiology, Ghent University (indicated by R-number). AB: Antibiotic resistance tested (+) or not (blanc). FU: GI-tract survival capacity and hydrophobicity tested (+) or not (blanc). More information on the test results can be found in Chapter 4 of this thesis.

Isolate	R-number	Identity (% of ID-database match)	Producttype	AB	FU
01A(28)	R10704	*Enterococcus faecium* (93.6%)	Powder	+	
01B(28)	R10684	*Enterococcus faecium* (95.1%)	Powder	+	
01C(28)	R10705	*Enterococcus faecium* (92.9%)	Powder	+	
01D(28)	R17051	*Enterococcus faecium* (95.3%)	Powder	+	
05A(28)	R11648	*Lactobacillus lindneri-like* (80%)	Capsules		
06A(28)	R11400	*Bacillus cereus* (ARDRA)	Capsules		
06B(28)	R11401	*Bacillus cereus* (ARDRA)	Capsules		
07A(37)	R11402	*Lactobacillus rhamnosus* (96.4%)	Capsules	+	
07B(37)	R10688	*Lactobacillus rhamnosus* (95.7%)	Capsules	+	
07C(37)	R11403	*Lactobacillus rhamnosus* (96.9%)	Capsules	+	
09A(28)	R11404	*Enterococcus faecium* (95.2%)	Capsules	+	
09B(28)	R17050	*Enterococcus faecium* (94.9%)	Capsules	+	
09C(28)	R11644	*Enterococcus faecium* (96.3%)	Capsules	+	
09D(28)	R11643	*Enterococcus faecium* (96.3%)	Capsules	+	
10C(28)	R11652	*Enterococcus faecium* (93.0%)	Capsules		
11A(28)	R12679	*Enterococcus faecium* (94.4%)	Capsules	+	
11A(37)	R11406	*Enterococcus faecium* (94.8%)	Capsules	+	
11B(28)	R12680	*Enterococcus faecium* (95.7%)	Capsules	+	
11B(37)	R11407	*Enterococcus faecium* (92.6%)	Capsules	+	
11C(37)	R11408	*Enterococcus faecium* (96.1%)	Capsules	+	
11D(37)	R11409	*Enterococcus faecium* (96.3%)	Capsules	+	
12A(28)	R10687	*Enterococcus faecium* (95.5%)	Capsules		
13A(28)	R10703	*Enterococcus faecium* (94.4%)	Powder	+	
13A(37)	R11410	*Enterococcus faecium* (95.7%)	Powder	+	
13B(28)	R10702	*Enterococcus faecium* (95.2%)	Powder	+	
13B(37)	R11411	*Enterococcus faecium* (95.8%)	Powder	+	
13C(28)	R12681	*Enterococcus faecium* (93.4%)	Powder	+	
13C(37)	R11412	*Enterococcus faecium* (95.1%)	Powder	+	
13D(28)	R12682	*Enterococcus faecium* (94.6%)	Powder	+	
13D(37)	R11413	*Enterococcus faecium* (96.8%)	Powder	+	+
13E(28)	R12683	*Enterococcus faecium* (94.1%)	Powder	+	
13E(37)	R11702	*Enterococcus faecium* (90.0%)	Powder		
13F(28)	R12684	*Enterococcus faecium* (95.2%)	Powder	+	
13F(37)	R11703	*Enterococcus faecium* (91.0%)	Powder		
13G(37)	R11704	*Enterococcus faecium* (92.2%)	Powder		
14A(28)	R10693	*Lactobacillus plantarum* (95.7%)	Powder	+	
14A(37)	R11414	*Lactobacillus plantarum* (94.1%)	Powder	+	
14B(28)	R10694	*Lactobacillus plantarum* (93.0%)	Powder		
14B(37)	R10692	*Lactobacillus plantarum* (95.4%)	Powder	+	+

Isolate	R-number	Identity (% of ID-database match)	Producttype	AB	FU
14C(28)	R10695	*Lactobacillus plantarum* (94.0%)	Powder		
14D(37)	R11705	*Lactobacillus plantarum* (93.1%)	Powder		
14F(37)	R11707	*Lactobacillus plantarum* (92.9%)	Powder		
17A	R15647	*Lactobacillus reuteri* (93.7%)	Powder	+	+
17C	R17095	*Lactobacillus reuteri* (93.8%)	Powder	+	
17D	R17092	*Lactobacillus reuteri* (91.2%)	Powder	+	
24A(28)	R10689	*Lactobacillus paracasei paracasei* (96.0%)	Capsules		
24A(37)	R10691	*Lactobacillus paracasei paracasei* (96.2%)	Capsules	+	
24B(28)	R10690	*Lactobacillus paracasei paracasei* (96.1%)	Capsules	+	
24B(37)	R11417	*Lactobacillus paracasei paracasei* (95.7%)	Capsules	+	
26A(28)	R10696	*Pediococcus acidilactici spp.1 (*95.1%)	Capsules	+	
26A(37)	R12685	*Bifidobacterium lactis* (94.3%)	Capsules		
26B(28)	R10685	*Pediococcus acidilactici spp.1*(91.6%)	Capsules	+	
26B(37)	R11708	*Bifidobacterium lactis* (94.9%)	Capsules		
26C(28)	R10686	*Lactobacillus plantarum* (95.1%)	Capsules	+	+
26C(37)	R12686	*Bifidobacterium lactis* (94.6%)	Capsules		
27A(28)	R10699	*Pediococcus acidilactici spp.1*(92.1%)	Capsules	+	
27A(37)	R10682	*Pediococcus acidilactici spp.1*(91.4%)	Capsules	+	
27B(28)	R10700	*Pediococcus acidilactici spp.1*(90.1%)	Capsules	+	
27C(28)	R10701	*Pediococcus acidilactici spp.1*(94.0%)	Capsules	+	
27C(37)	R10698	*Pediococcus acidilactici spp.1*(90.7%)	Capsules	+	
27D(28)	R12687	*Pediococcus acidilactici spp.1* (92.3%)	Capsules	+	
28A(28)	R11419	*Lactobacillus rhamnosus* (94.6%)	Capsules		
28A(37)	R11420	*Lactobacillus rhamnosus* (95.0%)	Capsules	+	
28B(28)	R10683	*Lactobacillus rhamnosus* (95%)	Capsules	+	
28B(37)	R11421	*Lactobacillus rhamnosus* (95.9%)	Capsules	+	
29C	R17093	*Lactobacillus reuteri* (92.8%)	Tablet	+	
30A(28)	R11653	*Lactobacillus rhamnosus* (95.4%)	Dairy Drink	+	
30A(37)	R11647	*Lactobacillus rhamnosus* (94.9%)	Dairy Drink	+	
30B(28)	R11645	*Lactobacillus rhamnosus* (95.1%)	Dairy Drink	+	
30B(37)	R11649	*Lactobacillus rhamnosus* (94.9%)	Dairy Drink	+	
30C(28)	R11650	*Lactobacillus rhamnosus* (95.7%)	Dairy Drink	+	
30D(28)	R12688	*Lactobacillus rhamnosus* (95.5%)	Dairy Drink	+	
30E(28)	R12689	*Lactobacillus rhamnosus* (90.1%)	Dairy Drink	+	
30F(28)	R12690	*Lactobacillus rhamnosus* (90.2%)	Dairy Drink	+	
30G(28)	R12691	*Lactobacillus rhamnosus* (90.9%)	Dairy Drink	+	
31A(37)	R12692	*Lactobacillus paracasei paracasei* (91.5%)	Dairy Drink	+	
31B(37)	R12693	*Lactobacillus paracasei paracasei* (91.2%)	Dairy Drink	+	
31C(37)	R12694	*Lactobacillus paracasei paracasei* (92.5%)	Dairy Drink	+	
32A(37)	R12695	*Lactobacillus paracasei paracasei* (92.4%)	Dairy Drink	+	
32B(37)	R12696	*Lactobacillus paracasei paracasei* (91.7%)	Dairy Drink	+	
32C(37)	R12697	*Lactobacillus paracasei paracasei* (89.0%)	Dairy Drink	+	
32D(37)	R12698	*Lactobacillus paracasei paracasei* (88.9%)	Dairy Drink	+	
32E(37)	R12699	*Lactobacillus paracasei paracasei* (95.1%)	Dairy Drink	+	
32F(37)	R12700	*Lactobacillus paracasei paracasei* (94.0%)	Dairy Drink	+	
32G(37)	R12701	*Lactobacillus paracasei paracasei* (93.3%)	Dairy Drink	+	

Isolate	R-number	Identity (% of ID-database match)	Producttype	AB	FU
33A	R17116	*Streptococcus thermophilus* (90.0%)	Dairy Drink		
33C	R17085	*Lactobacillus johnsonii* (96.3%)	Dairy Drink	+	
33E	R15650	*Lactobacillus acidophilus* (95.7%)	Dairy Drink	+	+
34C	R17121	*Streptococcus thermophilus* (92.8%)	Dairy Drink	+	
34E	R16044	*Lactobacillus paracasei paracasei* (93.2%)	Dairy Drink		
34F	R17062	*Lactobacillus acidophilus* (95.7%)	Dairy Drink	+	
34G	R16046	*Lactobacillus paracasei paracasei* (95.3%)	Dairy Drink		
35B	R17061	*Lactobacillus acidophilus* (95.4%)	Dairy Drink		
35C	R17114	*Streptococcus thermophilus* (88.0%)	Dairy Drink		
35D	R17123	*Streptococcus thermophilus* (93.9%)	Dairy Drink	+	
36C	R17109	*Streptococcus thermophilus* (85.9%)	Dairy Drink	+	
37A	R17059	*Lactobacillus acidophilus* (95.2%)	Dairy Drink		
37B1	R17107	*Streptococcus thermophilus* (84.2%)	Dairy Drink	+	
37B2	R17056	*Lactobacillus acidophilus* (92.9%)	Dairy Drink	+	
37D	R17110	*Streptococcus thermophilus* (86.2%)	Dairy Drink	+	
37E	R17136	*Lactobacillus acidophilus* (96.3%)	Dairy Drink	+	
37F	R17064	*Lactobacillus acidophilus* (96.3%)	Dairy Drink	+	
38A	R17118	*Streptococcus thermophilus* (90.9%)	Dairy Drink		
39A	R17088	*Lactobacillus johnsonii* (96.7%)	Dairy Drink	+	
39B	R17089	*Lactobacillus johnsonii* (96.8%)	Dairy Drink	+	
39C	R17083	*Lactobacillus johnsonii* (95.8%)	Dairy Drink	+	
39D	R17084	*Lactobacillus johnsonii* (96.2%)	Dairy Drink	+	
39E	R17081	*Lactobacillus johnsonii* (95.4%)	Dairy Drink		
39F	R17087	*Lactobacillus johnsonii* (96.5%)	Dairy Drink	+	
40A	R15661	*Lactobacillus delbrueckii bulgaricus* (95.6%)	Dairy Drink	+	+
40B	R17072	*Lactobacillus delbrueckii bulgaricus* (94.1%)	Dairy Drink	+	
40D	R17071	*Lactobacillus delbrueckii bulgaricus* (93.7%)	Dairy Drink	+	
41D	R17086	*Lactobacillus johnsonii* (96.4%)	Dairy Drink	+	
41F	R17077	*Lactobacillus johnsonii* (94.0%)	Dairy Drink	+	
42E	R17069	*Lactobacillus crispatus* (93.8%)	Dairy Drink	+	
42F	R17068	*Lactobacillus crispatus* (93.7%)	Dairy Drink	+	
43E	R16063	*Lactobacillus paracasei paracasei* (94.9%)	Dairy Drink		
43B	R16062	*Lactobacillus paracasei paracasei* (94.1%)	Dairy Drink		
44A	R15652	*Streptococcus thermophilus* (88.9%)	Dairy Drink	+	+
44B	R17122	*Streptococcus thermophilus* (93.7%)	Dairy Drink	+	
44C	R17108	*Streptococcus thermophilus* (84.7%)	Dairy Drink		
45A	R17112	*Streptococcus thermophilus* (87.7%)	Dairy Drink	+	
45B	R17119	*Streptococcus thermophilus* (91.2%)	Dairy Drink	+	
45C	R17105	*Streptococcus thermophilus* (75.5%)	Dairy Drink	+	
45D	R17106	*Streptococcus thermophilus* (75.6%)	Dairy Drink	+	
46C	R17101	*Lactococcus lactis lactis* (92.7%)	Dairy Drink		
47A	R17104	*Lactococcus lactis lactis* (93.4%)	Dairy Drink	+	
47B	R17103	*Lactococcus lactis lactis* (93.1%)	Dairy Drink	+	
48A	R17124	*Streptococcus thermophilus* (94.9%)	Dairy Drink	+	
48B	R17117	*Streptococcus thermophilus* (90.0%)	Dairy Drink	+	
49A	R15653	*Lactobacillus rhamnosus* (93.8%)	Dairy Drink	+	+

Isolate	R-number	Identity (% of ID-database match)	Producttype	AB	FU
49B	R17060	*Lactobacillus acidophilus* (95.3%)	Dairy Drink	+	
49C	R16072	*Lactobacillus rhamnosus* (95.8%)	Dairy Drink		
49E	R17135	*Lactobacillus acidophilus* (94.2%)	Dairy Drink	+	
50A	R15654	*Lactococcus lactis lactis* (92.7%)	Dairy Drink	+	+
51A	R15655	*Lactobacillus rhamnosus* (94.5%)	Dairy Drink	+	+
51B	R16073	*Lactobacillus rhamnosus* (93.3%)	Dairy Drink		
51C	R16074	*Lactobacillus rhamnosus* (90.9%)	Dairy Drink		
51D	R17098	*Lactobacillus rhamnosus* (94.3%)	Dairy Drink	+	
52D	R17120	*Streptococcus thermophilus* (91.2%)	Dairy Drink	+	
52E	R15656	*Lactobacillus johnsonii* (95.6%)	Dairy Drink	+	+
53A	R15657	*Lactobacillus crispatus* (94.5%)	Tablet	+	+
53C	R16076	*Lactobacillus rhamnosus* (89.9%)	Tablet		
53D	R17096	*Lactobacillus rhamnosus* (91.7%)	Tablet	+	
53F	R16077	*Lactobacillus rhamnosus* (94.5%)	Tablet		
54A	R17057	*Lactobacillus acidophilus* (93.4%)	Dairy Drink	+	
54B	R17055	*Lactobacillus acidophilus* (92.1%)	Dairy Drink	+	
54F	R17058	*Lactobacillus acidophilus* (94.5%)	Dairy Drink	+	
55A	R17074	*Lactobacillus johnsonii* (90.6%)	Dairy Drink	+	
55B	R17075	*Lactobacillus johnsonii* (91.8%)	Dairy Drink	+	
55C	R15659	*Lactobacillus johnsonii* (94.6%)	Dairy Drink	+	+
55D	R17080	*Lactobacillus johnsonii* (94.7%)	Dairy Drink	+	
55E	R17076	*Lactobacillus johnsonii* (93.8%)	Dairy Drink	+	
55F	R17078	*Lactobacillus johnsonii* (94.2%)	Dairy Drink	+	
A14	R17140	*Streptococcus thermophilus* (91.2%)	Dairy Drink		
A3	R17137	*Lactobacillus paracasei paracasei* (91.3%)	Dairy Drink		
A4	R17138	*Lactobacillus paracasei paracasei* (94.4%)	Dairy Drink		
A9	R17139	*Streptococcus thermophilus* (87.9%)	Dairy Drink		
B1	R17141	*Lactococcus lactis ssp. lactis* (90.0%)	Dairy Drink		
B10	R17144	*Lactobacillus delbrueckii ssp bulgaricus* (91.1%)	Dairy Drink		
B11	R17145	*Streptococcus thermophilus* (89.6%)	Dairy Drink		
B12)	R17146	*Lactobacillus delbrueckii ssp bulgaricus* (92.2%)	Dairy Drink		
B13)	R17147	*Bifidobacterium lactis* (93.8%)	Dairy Drink		
B13B2	R16038	*Enterococcus faecium* (92.1%)	Powder	+	
B13C	R17048	*Enterococcus faecium* (93.4%)	Powder	+	
B13D	R15646	*Lactococcus lactis lactis* (92.5%)	Powder	+	+
B13E	R14564	*Enterococcus faecium* (94.3%)	Powder	+	
B13F	R17049	*Enterococcus faecium* (94.7%)	Powder	+	
B32C	R15649	*Lactobacillus paracasei paracasei* (92.7%)	Dairy Drink	+	+
B4	R17142	*Streptococcus thermophilus* (87.0%)	Dairy Drink		
B42A	R17070	*Lactobacillus crispatus* (94.0%)	Dairy Drink	+	
B42C	R17066	*Lactobacillus crispatus* (92.8%)	Dairy Drink	+	
B42E	R17067	*Lactobacillus crispatus* (93.1%)	Dairy Drink	+	
B5A	R17113	*Streptococcus thermophilus* (87.9%)	Capsules	+	
B5B	R17111	*Streptococcus thermophilus* (87.3%)	Capsules	+	
B5E	R17054	*Lactobacillus acidophilus* (90.8%)	Capsules	+	
B5G	R17134	*Bifidobacterium lactis* (93.3%)	Capsules		

Isolate	R-number	Identity (% of ID-database match)	Producttype	AB	FU
B9	R17143	*Bifidobacterium lactis* (91.1%)	Dairy Drink		
C3	R17148	*Lactobacillus paracasei paracasei* (96.0%)	Dairy Drink		
C31A	R16040	*Lactobacillus paracasei paracasei* (94.9%)	Dairy Drink		
C31B	R17091	*Lactobacillus paracasei paracasei* (95.6%)	Dairy Drink	+	
C31C	R15648	*Lactobacillus paracasei paracasei* (95.0%)	Dairy Drink	+	+
C4	R17149	*Lactobacillus paracasei paracasei* (91.8%)	Dairy Drink		

Table 2: Strains used to construct DGGE identification databases. The LMG number indicates the accession number of the strains in the BCCM™/LMG culture collection.

Species	LMG number	Species	LMG number
Bacillus clausii	17945	*Bacillus coagulans*	6326T
Bacillus subtilis	7135T	*Bacillus cereus*	6923T
Bifidobacterium adolescentis	10733	*Bifidobacterium adolescentis*	11579
Bifidobacterium adolescentis	18898	*Bifidobacterium adolescentis*	10734
Bifidobacterium adolescentis	18897	*Bifidobacterium lactis*	11580
Bifidobacterium angulatum	11039T	*Bifidobacterium angulatum*	11568
Bifidobacterium angulatum	10503T	*Bifidobacterium minimum*	11592T
Bifidobacterium animalis	17135T	*Bifidobacterium animalis*	10508T
Bifidobacterium animalis	18900	*Bifidobacterium animalis*	11083T
Bifidobacterium asteroides	10735T	*Bifidobacterium asteroides*	11581
Bifidobacterium bifidum	11041T	*Bifidobacterium bifidum*	11583
Bifidobacterium bifidum	11582	*Bifidobacterium bifidum*	13195
Bifidobacterium bifidum	13200	*Bifidobacterium cuniculi*	10738T
Bifidobacterium boum	21815T	*Bifidobacterium gallinarum*	11586T
Bifidobacterium breve	10645	*Bifidobacterium breve*	11040
Bifidobacterium breve	11613	*Bifidobacterium breve*	11084
Bifidobacterium breve	13208T	*Bifidobacterium breve*	13194
Bifidobacterium catenulatum	11043T	*Bifidobacterium catenulatum*	18894
Bifidobacterium choerinum	10510T	*Bifidobacterium saeculare*	14325T
Bifidobacterium coryneforme	18911T	*Bifidobacterium boum*	10736T
Bifidobacterium dentium	11045T	*Bifidobacterium dentium*	10507T
Bifidobacterium dentium	11585	*Bifidobacterium gallicum*	11596T
Bifidobacterium infantis	13204	*Bifidobacterium infantis*	11570
Bifidobacterium infantis	18901	*Bifidobacterium infantis*	11588
Bifidobacterium infantis	18902	*Bifidobacterium infantis*	8811T
Bifidobacterium lactis	18314T	*Bifidobacterium lactis*	18906
Bifidobacterium lactis	11615	*Bifidobacterium lactis*	18905
Bifidobacterium longum	18899	*Bifidobacterium longum*	11589
Bifidobacterium longum	13196	*Bifidobacterium longum*	13197T
Bifidobacterium magnum	11591T	*Bifidobacterium magnum*	11590
Bifidobacterium merycicum	11341T	*Bifidobacterium indicum*	11587T
Bifidobacterium pseudocatenulatum	18903	*Bifidobacterium pseudocatenulatum*	11593
Bifidobacterium pseudocatenulatum	18904	*Bifidobacterium pseudocatenulatum*	18910
Bifidobacterium pseudolongum subsp. *globosum*	11569T	*Bifidobacterium pseudolongum* subsp. *globosum*	11571T
Bifidobacterium pseudolongum subsp. *globosum*	11614	*Bifidobacterium pseudolongum* subsp. *pseudolongum*	11595
Bifidobacterium pseudolongum subsp. *pseudolongum*	11594	*Bifidobacterium adolescentis*	10502T
Bifidobacterium ruminantium	12588T	*Bifidobacterium ruminantium*	21811T
Bifidobacterium ruminantium	18896	*Bifidobacterium longum*	11047
Bifidobacterium scardovii	21589T	*Bifidobacterium scardovii*	21590
Bifidobacterium subtile	11597T	*Bifidobacterium pseudocatenulatum*	10505T

Species	LMG number	Species	LMG number
Bifidobacterium suis	18891	*Bifidobacterium suis*	21814T
Bifidobacterium thermacidophilum	21396	*Bifidobacterium thermacidophilum*	21397
Bifidobacterium thermacidophilum	21395T	*Bifidobacterium pullorum*	21816T
Bifidobacterium thermophilum	11574	*Bifidobacterium thermophilum*	11573T
Bifidobacterium thermophilum	18893	*Bifidobacterium thermophilum*	21813T
Bifidobacterium thermophilum	11599	*Bifidobacterium thermophilum*	18892
Enterococcus faecalis	7937T	*Enterococcus faecalis*	14206
Enterococcus faecium	11423T	*Enterococcus faecium*	14204
Lactobacillus acidophilus	9433T	*Lactobacillus acidophilus*	11466
Lactobacillus casei	13087T	*Lactobacillus casei*	8152
Lactobacillus crispatus	9479T	*Lactobacillus crispatus*	12003
Lactobacillus delbreuckii subsp. *lactis*	7942T	*Lactobacillus salivarius*	9477
Lactobacillus delbrueckii subsp. *bulgaricus*	6901T	*Lactobacillus delbrueckii* subsp. *bulgaricus*	12168
Lactobacillus fermentum	6902T	*Lactobacillus gallinarum*	9435T
Lactobacillus gasseri	9203T	*Lactobacillus paracasei*	13717
Lactobacillus helveticus	6413T	*Lactobacillus reuteri*	9213T
Lactobacillus johnsonii	9436T	*Lactobacillus johnsonii*	11468
Lactobacillus plantarum	6907T	*Lactobacillus plantarum*	18023
Lactobacillus rhamnosus	6400T	*Lactobacillus rhamnosus*	18030
Lactococcus lactis subsp. *Lactis*	6890T	*Pediococcus acidilactici* subsp. *acidilactici*	11384
Propionibacterium freundenreichii	11572	*Gardnerella vaginalis*	7832T
Streptococcus thermophilus	6896T	*Streptococcus thermophilus*	11164

Curriculum Vitae

1. General Information

Name: Robin D.H. Temmerman
Gender: Male
Date of birth: 20 December 1976
Place of birth: Lokeren (Belgium)
Address: Zelestraat 48W6, B-9160 Lokeren

2. Education

1982 - 1988: Primary School: Rijksbasisschool Noord, Lokeren

1988 - 1994: Secondary School: Sint-Lodewijkscollege, Lokeren
> Science type B

1994 - 2003: University: Ghent University

> 1994 - 1997: Biology
> Bachelor in Biology

> 1997 - 1999: Biotechnology
> Master in Biotechnology

> Dissertation: “Isolation, characterisation and identification of oxytetracycline-resistant bacteria originating from a Belgian fish farm.”

> 1999 - 2003: Ph.D in Biochemistry:

> **“Culture-dependent and culture-independent microbial analysis of probiotics.”**

3. Additional information

3.1. Publications in international journals (peer-reviewed)

- Huys, G., Gevers, D., Temmerman, R., Cnockaert, M., Denys, R., Rhodes, G., Pickup, R., McGann, P., Hiney, M., Smith, P., Swings, J. (2001). Comparison of the antimicrobial tolerance of oxytetracycline-resistant heterotrophic bacteria isolated from hospital sewage and freshwater fishfarm water in Belgium. *Systematic and Applied Microbiology*. **24:**122-130

- Temmerman, R., Pot, B., Huys, G., Swings, J. (2003). Identification and antibiotic susceptibility of bacterial isolates from probiotic products. *International Journal of Food Microbiology*. **81(1):**1-10.

- Temmerman, R., Scheirlinck, I., Huys, G. and Swings, J. (2003). Culture-independent Analysis of Probiotic Products using Denaturing Gradient Gel Electrophoresis (DGGE). *Applied and Environmental Microbiology* **69(1):**220-226.

- Temmerman, R., Masco, L., Vanhoutte, T., Huys, G. and Swings, J. (2003). Development and Validation of a Nested PCR- Denaturing Gradient Gel Electrophoresis Method for Taxonomic Characterization of Bifidobacterial Communities. *Applied and Environmental Microbiology.* **In press.**

- Temmerman, R. Huys, G. and Swings, J. (2003). Identification and detection of food-associated Lactic Acid Bacteria: An overview of culture-dependent and culture-independent methods. *Trends in Food Science and Technology.* **Submitted.**

- Temmerman, R. Masco, L., Huys, G. and Swings J. (2004). Enumeration and Identification of Probiotics. In: Probiotics in Food Safety and Human Health (I. Goktepe & V. Juneja, Eds.). Marcel Dekker Inc., New York. **Submitted**.

3.2. Publications in international journals (not peer-reviewed)

- Temmerman R., Huys G., Pot B. and Swings J. (2002). Quality analysis and label correctness of commercial probiotic products. *Innovations in Food Technology* **14**:72-73.

- Temmerman R. and Masco L. (2003). Probiotics: Health aspects and applications. *Innovations in Food Technology* **18:**16-18.

3.3. Lectures at international conferences

- Temmerman, R., Pot, B., Huys, G. and Swings, J. (2001). *A quality analysis of commercial probiotic products.* 15th Forum for Applied Biotechnology. 24-25 September 2001, Het Pand, Ghent, Belgium

- Temmerman, R., Masco, L. and Swings, J. (2003). *Potential of DGGE for the identification of lactic acid bacteria.* New Functional Ingredients and Foods (NFIF 2003). 9-11 April 2003, Copenhagen, Denmark.

- Temmerman, R. (2003). *Quality of probiotic products.* BioGaia Symposium. 1-3 June 2003, Stockholm, Sweden.

3.4. Stay in foreign labs

- 9 – 11 February 2000: Laboratory of Microbiology, Universiteit Wageningen. Prof. W. de Vos and Dr. A. Akkermans. Comparative study between TGGE and DGGE.

3.5. Attended courses

- Analysis of Biochemical, Molecular and Supramolecular Processes in Cells by Advanced Fluorescent Techniques. Prof. Dr. J. Grooten. Special PhD course Biotechnology 2000. Ghent University.

- Functional Foods: Quo vadis? Course 2001, Ingenieurshuis – KVIV, Antwerp.

- Molecular Techniques in Environmental Microbiology. Prof. Dr. Ir. E. Top. Post graduate course, KaHo Sint-Lieven, April 2001.

- Wecom communication course. Written module. Prof. J. Huypens. Oral module. Bob de Groof. January - March 2002. Ghent University.

3.6. Miscellaneous

- August 2001 - July 2002: Supervision of a student making her dissertation to obtain the degree of Master in Biotechnology. “Optimization of DGGE for the analysis of probiotic products.” by Ilse Scheirlinck. Promotor Prof. Jean Swings.

- December 2000 and 2001: Assistant at the practical course of Microbiology for the Master’s students of Biology, Biochemistry and Biotechnology.

- February 2003: Head-assistant at the practical course of Microbiology for the Master’s students of Biology, Biochemistry and Biotechnology.